

REMARKS

The new claims submitted above find support throughout the specification as filed. No prohibited new matter has been added. Exemplary support is provided in the table below:

New Claims	Support in Specification
76-77	page 13, lines 5-15; page 16, line 3 to page 17, line 8; page 20, lines 3-20; page 32, line 23-page 33, line 9; page 36, lines 2-6; page 37, lines 17-20; Examples 10-11
78-79	page 13, lines 7-8
80-81	page 33, lines 7- 9
82-84	page 14, lines 10-16
85-89	page 22, lines 8-10
90-91	page 30, lines 6-10
92-103	page 28, lines 14-23; page 36, line 2 to page 37, line 16; Example 11; page 88, lines 11-14
104-114	Example 12
115-116	original claim 1; page 4, line 14; page 19, lines 4-25; page 44, line 6, to page 45, line 2; page 48, lines 22-25
117-118	page 4, line 12; page 45, lines 23-25
119-120	page 4, line 12; page 7, lines 4-9; page 45, lines 3-23
121-122	page 48, lines 7-24
123-125	page 48, line 24, to page 49, line 4
126-140	original claims 5-22

Applicants also wish to respond in part to the rejection under 35 U.S.C. §103(a) based on Fire in view of Wianny, which was set forth in the Office Action mailed June 3, 2004, in parent application 10/062,707. On page 12 of the Office Action, the Examiner stated: “Fire *et al.* are deficient in that they do not specifically teach the use of conditions that inhibit or prevent an interferon response. However, Wianny *et al.* teach . . . the use of conditions that will prevent or inhibit an interferon response in animal cells.” The Examiner made particular reference to the discussion in Wianny *et al.*, stating “we have shown here [using our conditions] that the injection of a dsRNA . . . does not cause a general translational arrest [i.e., no interferon response that would otherwise lead to apoptosis], because embryos continue to develop and we see no signs of cell death.” Thus, importantly, the teachings of Wianny *et al.* are all directed to RNA interference in vertebrate embryo cells, more specifically, mouse embryonic cells.

As known to those of skill in the art, the “interferon response” discussed in the Office Action is also typically known as the “PKR response” or “stress response.” This response was initially observed as a response to certain viruses, since dsRNAs are a common intermediate or end product in many viral infections. To elaborate for the record, the introduction of long dsRNAs into the cytoplasm of a stress-competent cell induces the synthesis of alpha and beta interferons. Protein kinase R (PKR), which is normally present in an inactive form at low levels in cells, is induced by interferon. When dsRNAs enter the cells, they are thought to bind to PKR and induce dimerization, resulting in a conformational change, the unmasking of a catalytic domain, and autophosphorylation. After PKR is activated, it can phosphorylate a number of substrates, including eukaryotic initiation factor 2 (eIF-2 α). Phosphorylated eIF-2 α then binds to eIF2B, which impairs the eIF2B-catalyzed guanine nucleotide exchange reaction, resulting in inhibition of protein synthesis. (See Thomis and Samuel, 1992, PNAS 89: 10837-41, at p.

10837, col. 1; see also Wang and Carmichael, 2004, Microbiol. Mol. Biol. Rev. 68(3): 432-52 for a more recent review on the interferon/PKR response to dsRNA) (copies attached).

Applicants respectfully note that Wianny *et al.* do not teach the use of conditions that will inhibit or prevent an interferon response because vertebrate embryonic cells are naturally deficient in the interferon response. Moreover, it has been known in the art since at least the 1970s that vertebrate embryonic cells are deficient in the interferon response.

For instance, as stated in Haggarty *et al.* (1988, Nucleic Acids Res. 16(22): 10575-92, copy attached), one of the markers associated with the undifferentiated state of embryonal carcinoma (EC) cells is "the inability to produce interferon in response to exposure to viruses or double stranded RNA, a characteristic shared with early embryos" (with emphasis). In fact, Haggarty *et al.* cites several articles published between 1978-1984 that show that, even then, it was known that vertebrate embryonic cells do not produce or respond to interferon. See Burke, 1978, Cell 13:243-48; Coveney *et al.*, 1984, Biochem. Biophys. Res. Commun. 121: 290-96; Barlow *et al.*, 1984, Differentiation 27:229-35 (copies attached).

Thus, Wianny *et al.* did not overcome the prejudice in the art concerning the use of RNAi in vertebrate cells, because Wianny *et al.* did not prevent or inhibit the interferon response as stated in the Office Action. Rather, Wianny *et al.* merely show the practice of RNAi in vertebrate cells that were already known in the art to be deficient in the interferon response.

In contrast to Wianny *et al.*, Applicants have in fact overcome the prejudice in the art regarding the use of RNAi in vertebrate, and particularly mammalian, cells. As reflected in new claims 76-102 submitted above, Applicants believe that they are the first to disclose the successful practice of RNAi in stress response-competent vertebrate cells. In particular, Applicants believe that they are the first to disclose that the stress response associated with

introducing double stranded RNA (dsRNA) into vertebrate cells can be avoided by expressing the dsRNA from an expression vector *in vivo*.

For example, as reported in the specification in Example 10, “No cellular toxicity was seen with any of the dsRNAs generated by the RNA expression vectors suggesting that cytoplasmic expression of dsRNA does not induce the interferon response” (page 79, lines 16-18; see also Applicants’ priority application 60/265,805, filed January 31, 2001, in the paragraph bridging pages 57-58, “No cellular toxicity was seen with any of the dsRNAs generated by the expression vectors, indicating that an interferon response is not induced.”). In Example 11, the present inventors confirmed these findings and showed that intracellular expression of dsRNA using an expression vector did not induce the RNA stress response. The cells used in the experiments of Example 11 were competent for RNA stress response induction as was demonstrated by the ability of cationic lipid complexed to poly(I)(C) or to *in vitro* transcribed dsRNA to induce/activate all tested components of this response. In addition, the cells were found to be responsive to exogenously added interferon. These results show that the cells used for these experiments were not defective in their ability to mount an RNA stress response and therefore can be used as predictors for other cells, both in cell culture and *in vivo* in animal models. (See Example 11, page 80, lines 6-18; page 81, lines 19-21; and page 85, lines 1-12).

Prior to Applicants’ disclosure, the skilled artisan working in the RNAi field would not have believed that specific inhibition of gene expression could be accomplished in stress response-competent vertebrate cells using dsRNA. This is evidenced by the attached article by Montgomery and Fire (TIG 1998, 14(7): 255-58), in which the authors state, “Any gene-specific interference by dsRNA in PKR-proficient mammalian cells would be dependent on a transient lapse in the PKR response, or on a controlled level of dsRNA that was incapable of

activatingPKR” (p. 258, col. 2). In particular, the skilled artisan would not have believed that specific inhibition of gene expression could be accomplished in stress response-competent vertebrate cells using long dsRNA that is over 30 base pairs long. This is evidenced by several articles published after Applicants’ priority date of January 31, 2001, which show that those of skill in the field of RNA interference did not believe that RNAi could be accomplished in vertebrate cells that were capable of mounting an interferon response.

For example, the attached article by Billy *et al.*, published in December 2001, shows that those of skill in the RNAi field at that time believed that RNAi was a eukaryotic phenomenon, that both long and short dsRNA molecules could be used to produce RNAi in invertebrate animals, and that long dsRNAs produce non-specific toxicity in differentiated vertebrate cells due to the interferon response (see Introduction section on page 14428-33). In fact, based on the disclosure of Billy *et al.*, the skilled artisan would have believed that RNAi in vertebrate cells is only possible where the cells are deficient in the interferon response. This prejudice is reiterated by Diallo *et al.* in 2003 (attached), who provided one of the first reports in a scientific journal that long, endogenously expressed dsRNA can be used to mediate RNAi in mammalian cells in the absence of an interferon response (see particularly the discussion at page 390).

Applicants have overcome this prejudice in the art concerning the use of dsRNAs, and particularly long dsRNAs, to mediate RNA interference in vertebrate cells. To Applicants’ knowledge, they were the first to describe the successful practice of RNAi in stress response-competent vertebrate cells using endogenously expressed dsRNA.

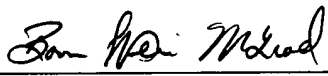
In view of the above newly submitted claims and remarks, prompt and judicious examination, and early indication of allowable subject matter, are respectfully requested.

Except for issue fees payable under 37 CFR §1.18, the commissioner is hereby authorized by this paper to charge any additional fees during the pendency of this application including fees due under 37 CFR §1.16 and 1.17 which may be required, including any required extension of time fees, or credit any overpayment to Deposit Account 50-0310. This paragraph is intended to be a **CONSTRUCTIVE PETITION FOR EXTENSION OF TIME** in accordance with 37 CFR §1.136(a)(3).

If the Examiner has any further questions relating to this Reply or to the application in general, he is respectfully requested to contact the undersigned by telephone so that allowance of the present application may be expedited.

Respectfully submitted,

Date: September 28, 2006

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Mechanism of interferon action: Autoregulation of RNA-dependent P1/eIF-2 α protein kinase (PKR) expression in transfected mammalian cells

(translational control/protein synthesis factor eIF-2 α /kinase phosphotransfer mutant/RNA-binding protein)

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ABSTRACT The expression of a molecular cDNA clone (P1 KIN) of the human RNA-dependent P1/eIF-2 α protein kinase (PKR) was examined in transfected monkey cells and in cell-free protein-synthesizing systems. Expression of the wild-type (wt) P1 KIN cDNA, which encodes an active protein kinase, was compared with that of the phosphotransfer catalytic domain II Lys-296 \rightarrow Arg (K296R) mutant cDNA, which does not encode an active kinase. wt and K296R mutant P1 mRNAs prepared by transcription *in vitro* with T7 RNA polymerase programmed the cell-free synthesis of P1 ribosome-associated protein with comparable efficiency in the rabbit reticulocyte system. The K296R mutant P1 protein was also efficiently synthesized *in vivo* in transfected COS monkey cells. However, synthesis of the wt P1 protein was reduced about 30-fold in transfected COS cells as compared with the K296R mutant P1 protein. Cotransfection of wt P1 KIN cDNA with either K296R mutant P1 KIN cDNA or reovirus S4 cDNA greatly reduced the synthesis of K296R mutant P1 protein and reovirus σ 3 protein, respectively. Although the wt and K296R mutant P1 KIN plasmid expression vectors replicated with comparable efficiencies in COS cells, the steady-state amount of P1 mRNA was about 3-fold less in COS cells transfected with the wt as compared with the K296R mutant P1 KIN cDNA. These results suggest that RNA-dependent P1 protein kinase expression is autoregulated *in vivo* in transfected mammalian cells primarily at the level of translation by a mechanism that is likely dependent upon catalytically active P1 kinase.

Among the enzymes induced by interferon (IFN) is a protein-serine/threonine kinase, designated the P1/eIF-2 α protein kinase, but also known as the P1 kinase, p68 kinase, DAI, dsI, and PKR (1, 2). The IFN-induced P1/eIF-2 α kinase is dependent upon RNA for activation, a process which involves an RNA-dependent autophosphorylation of P1 ribosomal protein (3-6). When activated, the P1/eIF-2 α kinase catalyzes the phosphorylation of the α subunit of protein synthesis initiation factor eIF-2 at Ser-51 (7, 8). Phosphorylation of eIF-2 α causes an inhibition of protein synthesis at the initiation step of translation (9). Considerable evidence has accumulated consistent with the notion that the P1/eIF-2 α kinase plays a central role in translational control and in the antiviral action of IFN (2, 10).

Molecular cDNA clones of the P1/eIF-2 α protein kinase have been obtained from human amnion U cells (11) and human Daudi cells (12, 13). The deduced amino acid sequence of the cDNA predicts a 551-amino acid protein. The catalytic domains conserved among protein-serine/threonine kinases are present within the C-terminal half of the P1 protein. Transcripts prepared from cDNA clones of the

kinase program the cell-free synthesis of a 67-kDa protein which possesses P1/eIF-2 α protein kinase activity (12) and which is indistinguishable by immunoprecipitation and immunoblot gel analyses from authentic protein P1 synthesized in IFN-treated human cells (11). P1 protein synthesized from the cDNA clone P1 KIN, both in cell-free systems and in *Escherichia coli*, possesses RNA-binding activity which maps by deletion analysis to a domain within the N-terminal 98 residues of P1 (13-15). In contrast to the information gained from studying the expression of the P1 KIN cDNA in rabbit reticulocyte lysates and in bacteria, no information is yet available concerning the expression of P1 KIN cDNA in animal cells.

We report herein the expression of wild-type (wt) and mutant forms of the P1 KIN cDNA in transfected mammalian cells. The results suggest that the synthesis of wt P1 protein is autoregulated principally at the level of translation, whereas the synthesis of a catalytic domain II mutant P1 protein [Lys-296 \rightarrow Arg (K296R)] deficient in kinase activity is not autoregulated.

EXPERIMENTAL PROCEDURES

Expression Plasmid Constructions. The molecular cloning of the RNA-dependent P1/eIF-2 α protein kinase from human amnion U cells and the consensus sequence of the U cell full-length P1 KIN cDNA (GenBank accession no. M85294) have been previously reported (11). The general-purpose expression vector pJC119 (16) was used to express the P1 KIN cDNA within transfected COS cells by a scheme similar to that utilized to express full-length cDNA copies of reovirus S-class genes (17).

Plasmid vector pSVP1KIN(Wt) containing the complete coding region of the wt P1 protein was constructed by ligation of the 1.8-kilobase (kb) *Hind*III/*Pst* I P1 fragment from pBlue-P1KIN(Wt) (11) into the *Bam*HI site of the pJC119 expression vector. The ends of the *Hind*III/*Pst* I P1 fragment were blunted by using the Klenow fragment of *E. coli* DNA polymerase (for *Pst* I, incubation was overnight at 16°C without nucleotides, and for *Hind*III incubation was for 1 hr at 37°C with nucleotides), and the *Bam*HI-digested pJC119 vector was likewise blunted with the Klenow fragment prior to ligation.

Plasmid vector pSVP1KIN(K296R) containing the complete coding region of a K296R mutant P1 protein was constructed by replacing the *Bgl* II fragment of pSVP1KIN(Wt) with the *Bgl* II fragment isolated from plasmid pBlue-P1KIN(K296R). The correct orientation was confirmed by

restriction analysis, and the presence of the mutation (K296R) was verified by direct sequence analysis.

Plasmid vectors pSVS1 and pSVS4 containing full-length cDNAs of the reovirus serotype 1 Lang strain wt *S1* gene (GenBank accession no. M14779) and *S4* gene (GenBank accession no. M13139) were previously described (17).

Oligonucleotide-Directed Mutagenesis. The point mutant of P1 protein kinase, K296R, was prepared by site-directed mutagenesis. Briefly, the *Bgl* II (721)/*Eco*RI (1099) fragment of the P1 KIN cDNA was subcloned in the pBluescript II SK (+) vector (Stratagene), and uracil-containing single-stranded template DNA was prepared by using *E. coli* CJ236 and the helper phage M13K07. The mutant oligonucleotide 5'-ATTTAACACGCTCTAATAACG-3' (minus-sense nucleotides 898–879), synthesized by using an Applied Biosystems model 380A automated DNA synthesizer, was annealed with the plus-sense template single-stranded DNA, extended with T7 DNA polymerase, and ligated with T4 DNA ligase. The resulting double-stranded DNA plasmid was used to transform *E. coli* MV1190; recombinants were directly sequenced by the dideoxynucleotide chain-termination method (18). The *Acc* I/*Bcl* I fragment containing the Lys-296 → Arg mutation was then introduced into the full-length pBlue-P1 KIN cDNA clone by restriction fragment exchange, resulting in the plasmid pBlue-P1KIN(K296R).

Cell Maintenance and Transfection. COS African green monkey kidney cells were cultured in monolayer as previously described (17, 19). The pSVP1KIN, pSVS1, and pSVS4 expression constructs were introduced into COS cells by the DEAE-dextran/chloroquine phosphate transfection method (17, 19, 20). All transfections were performed with DNA at 5 µg/ml.

Measurement of Protein Synthesis *in Vivo*. The synthesis of P1 protein and reovirus $\sigma 1$ and $\sigma 3$ proteins was measured at 48 hr after transfection by pulse-labeling with [35 S]methionine for 60 min, preparation of cell-free extracts by lysis with buffer containing Nonidet P-40, immunoprecipitation with a saturating amount of rabbit polyclonal antibody and Formalin-fixed *Staphylococcus aureus*, and analysis of the proteins by NaDodSO₄/polyacrylamide gel electrophoresis and autoradiography (11, 19). Protein synthesis was quantitated by scanning autoradiograms with an LKB Ultrosan XL laser densitometer. The methods have previously been described in detail (11, 19, 21).

Immunoblot Analysis. Western immunoblots were prepared by the method of Towbin *et al.* (22) as previously described (11).

Northern Gel-Blot Analysis. Northern gel-blot analysis of RNA transcripts was carried out as previously described (11).

Southern Gel-Blot Analysis. Southern gel-blot analysis (23) was carried out on DNA from transfected COS cells isolated by a modification of the Hirt procedure (24).

Preparation of P1 mRNA and Translation *in Vitro*. P1 mRNA transcripts were prepared as *in vitro* transcription products by using T7 RNA polymerase and the pBluescript vector construction pBlue-P1 KIN containing the full-length P1 KIN cDNA insert (11, 25). The structural integrity of the RNA transcripts was ascertained by formaldehyde/agarose gel electrophoresis followed by autoradiography.

The *in vitro* translation of P1 mRNA catalyzed by rabbit reticulocyte lysates (Promega) and the analysis of [35 S]-labeled protein products were as previously described (25).

Materials. Unless otherwise specified, materials and reagents were as previously described (11, 14, 17).

RESULTS

Expression of the P1 KIN cDNA in Transfected Cells. To express the cDNA copy of the P1 protein kinase in mammalian cells, expression plasmids were constructed containing a

1.8-kb insert possessing the 551-amino acid open reading frame of the P1 KIN cDNA (11). The general-purpose eukaryotic expression vector pJC119 (16) was used, with P1 protein expression under the control of the simian virus 40 (SV40) late promoter (16).

P1 KIN expression constructs containing either the wt cDNA [pSVP1KIN(Wt)] or the catalytic domain residue 296 mutant cDNA [pSVP1KIN(K296R)] were introduced into COS monkey cells by the DEAE-dextran/chloroquine phosphate transfection method. Synthesis of P1 protein was then measured by pulse-labeling with [35 S]methionine. The K296R mutant P1 protein was efficiently synthesized in pSVP1KIN(K296R)-transfected cultures (Fig. 1A, lane 3). By contrast, the synthesis of wt P1 protein was far less efficient than that of the K296R mutant P1 protein (Fig. 1A, lanes 2 and 3).

Although the wt P1 protein was not efficiently synthesized in transfected COS cells, wt P1 protein was efficiently synthesized in rabbit reticulocyte lysates (Fig. 1B, lane 5). Furthermore, the wt P1 mRNA was translated *in vitro* with an efficiency comparable to that of the K296R mutant P1 mRNA (Fig. 1B, lane 5 as compared with lane 6). Quantitation of the amount of P1 protein synthesized *in vitro* at different mRNA concentrations, both in the presence and in the absence of the kinase antagonist 2-aminopurine, revealed less than a 2-fold difference in the translational efficiency between wt and K296R mutant P1 mRNAs (D.C.T., S. J. McCormack, J. P. Doohan, and C.E.S., unpublished observations).

The steady-state level of P1 protein was also examined in transfected cells by Western immunoblot analysis (Fig. 2). Rabbit immune serum, prepared against the recombinant P1

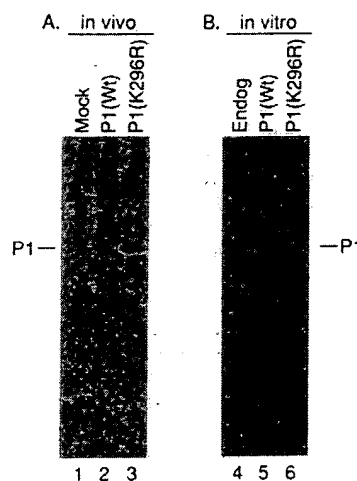


FIG. 1. Synthesis of recombinant wt and phosphotransfer K296R mutant P1 proteins *in vivo* and *in vitro*. (A) Synthesis of P1 protein in transfected COS cells. Monolayers of COS cells were transfected with the pSVP1KIN expression plasmids and 48 hr later cells were pulse-labeled with [35 S]methionine for 1 hr. Nonidet P-40 extracts were prepared, and P1 protein was immunoprecipitated and analyzed by NaDodSO₄/10% PAGE. Lane 1 (Mock), mock-transfected COS cells; lane 2 [P1(Wt)], wt pSVP1KIN(Wt)-transfected cells; and lane 3 [P1(K296R)], mutant pSVP1KIN(K296R)-transfected cells. (B) Synthesis of P1 protein in rabbit reticulocyte lysates. Capped P1 mRNAs, prepared from the pBlue-P1KIN and pBlue-P1KIN-(K296R) templates by transcription *in vitro*, were translated (4 µg/ml) by using the reticulocyte system, and the [35 S]methionine-labeled P1 protein product was immunoprecipitated and analyzed by NaDodSO₄/10% PAGE. Lane 4 (Endog), no exogenously added RNA; lane 5 [P1(Wt)], lysate programmed with wt P1 RNA; and lane 6 [P1(K296R)], lysate programmed with K296R mutant P1 RNA. The position of the 67-kDa P1 protein band is indicated.

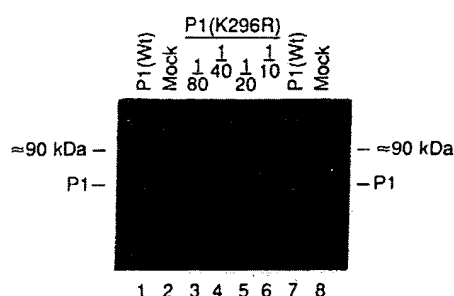


FIG. 2. Western immunoblot analysis of wt and K296R mutant P1 protein expressed in transfected COS cells. COS cells were transfected with the pSVP1KIN expression plasmids and unlabeled Nonidet P-40 extracts were prepared 48 hr later. Proteins were analyzed by NaDodSO₄/10% PAGE followed by Western immunoblotting (11, 22). Lanes 1 and 7 [P1(Wt)], wt pSVP1KIN(Wt)-transfected COS cells; lanes 2 and 8 (Mock), mock-transfected cells; lanes 3–6 [P1(K296R)], mutant pSVP1KIN(K296R)-transfected cells. Lanes 3, 4, 5, and 6 contained P1(K296R) extract diluted 1/80, 1/40, 1/20, and 1/10, respectively, relative to the amount loaded for the P1(Wt) lanes. The positions of the 67-kDa P1 protein band and a crossreacting ~90-kDa protein band (11) are indicated.

protein synthesized in *E. coli* (11), readily recognized a 67-kDa protein present in unlabeled extracts prepared from pSVP1KIN(K296R)-transfected COS cells (Fig. 2, lanes 3–6). The steady-state amount of the K296R mutant P1 protein that accumulated in transfected cells was about 30-fold greater than the amount of wt P1 protein that accumulated in transfected COS cells, as revealed from the quantitation of dilution standards by laser densitometry (Fig. 2, lanes 1, 4, 5, and 7).

The immune serum raised against recombinant human P1 protein also detected an endogenous monkey P1 protein in extracts prepared from mock-transfected COS cells (Fig. 2, lanes 2 and 8). However, the Western signal of the endogenous monkey P1 was weak, even compared with that obtained for the wt human P1 protein in transfected cells. Finally, P1 immune serum also recognized in monkey COS cells an ~90-kDa protein (Fig. 2) similar to that previously detected in human U cells. Preimmune serum did not recognize either protein P1 or the 90-kDa protein in U cells (11).

Effect of 2-Aminopurine on the Synthesis of P1 Protein in Transfected Cells. Among the inhibitors of P1/cIF-2 α protein kinase function are 2-aminopurine, a purine analogue, and σ 3, a reovirus RNA-binding protein (2).

Treatment of COS cells with 2-aminopurine beginning 24 hr after transfection did not enhance the rate of synthesis of either wt or K296R mutant P1 protein (Fig. 3, lanes 3–6). By contrast, as a positive control, treatment of pSVS1-transfected cells with 2-aminopurine increased the efficiency of reovirus σ 1 expression encoded by s1 mRNA (Fig. 3, lanes 7 and 8); s1 mRNA is an activator RNA of the P1 kinase (26). However, treatment of pSVS4-transfected cells with 2-aminopurine under identical conditions did not alter the efficiency of reovirus σ 3 expression (Fig. 3, lanes 9 and 10), as previously reported (19).

Although treatment of cells with 2-aminopurine beginning 24 hr after transfection did not affect either wt or K296R mutant P1 expression, when the 2-aminopurine treatment was begun only 3 hr after transfection and continued until the harvest at 48 hr, then the K296R mutant P1 expression was significantly reduced (Fig. 4, lane 4 versus lane 5). The expression of wt P1 was still not readily detected after a 45-hr treatment with 2-aminopurine (Fig. 4, lanes 2 and 3).

Expression of pSVP1KIN and pSVS4 Vectors in Cotransfected Cells. The reovirus σ 3 protein prevents the RNA-dependent activation of the P1 kinase by complexing with

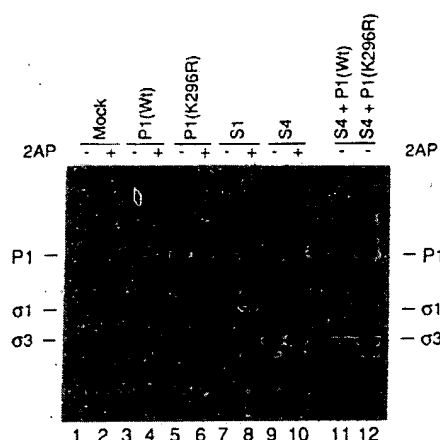


FIG. 3. Effect of 2-aminopurine and reovirus σ 3 protein on the expression of wt and K296R mutant P1 proteins in transfected COS cells. COS cells were transfected with pSVP1KIN vectors and [³⁵S]methionine-labeled as described for Fig. 1A. Where indicated, cells were treated with 10 mM 2-aminopurine (2AP), beginning 24 hr after transfection. P1 proteins and reovirus σ 1 and σ 3 proteins were immunoprecipitated and analyzed by NaDodSO₄/10% PAGE. Lanes 1 and 2 (Mock), mock-transfected COS cells; lanes 3 and 4 [P1(Wt)], pSVP1KIN(Wt)-transfected cells; lanes 5 and 6 [P1(K296R)], mutant pSVP1KIN(K296R)-transfected cells; lanes 7 and 8 (S1), pSVS1-transfected cells; lanes 9 and 10 (S4), pSVS4-transfected cells; lane 11 [S4 + P1(Wt)], cells cotransfected with pSVS4 and wt pSVP1KIN(Wt); and lane 12 [S4 + P1(K296R)], cells cotransfected with pSVS4 and mutant pSVP1KIN(K296R). The positions of proteins P1, σ 1, and σ 3 are indicated.

candidate activator RNAs (2, 27). However, cotransfection of the reovirus S4 gene (pSVS4) with the wt pSVP1KIN(Wt) vector surprisingly did not lead to an increase in the expression of the wt P1 protein (Fig. 3, lane 11). Rather, the synthesis of the reovirus σ 3 protein encoded by pSVS4 was somewhat decreased in the cultures cotransfected with pSVP1KIN(Wt)

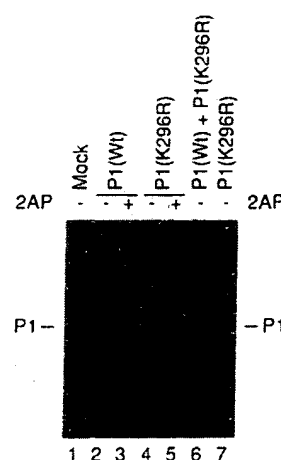


FIG. 4. Effect of 2-aminopurine and cotransfection on the expression of wt and K296R mutant P1 proteins in transfected COS cells. COS cells were transfected with the pSVP1KIN expression plasmids and the [³⁵S]-labeled P1 protein product was analyzed as for Fig. 3. Where indicated, cells were treated with 10 mM 2-aminopurine (2AP), beginning after chloroquine treatment. Lane 1 (Mock), mock-transfected COS cells; lanes 2 and 3 [P1(Wt)], wt pSVP1KIN(Wt)-transfected cells; lanes 4, 5, and 7 [P1(K296R)], mutant pSVP1KIN(K296R)-transfected cells; and lane 6 [P1(Wt) + P1(K296R)], cells cotransfected with wt pSVP1KIN(Wt) and mutant pSVP1KIN(K296R). The position of protein P1 is indicated.

(Fig. 3, lane 9 versus lane 11). Similarly, the synthesis of the K296R mutant P1 protein was inhibited in cultures cotransfected with pSVP1KIN(Wt) and pSVP1KIN(K296R) (Fig. 4, lanes 2, 4, and 6). Cotransfection of the mutant pSVP1KIN(K296R) vector with the reovirus S4 gene did not affect reovirus $\sigma 3$ synthesis, in contrast to the result obtained with the wt pSVP1KIN(Wt) cotransfection (Fig. 3, lanes 9, 11, and 12). These results suggest that the cellular localization of P1 protein or the affinity of P1 protein for activator RNAs, or both, may differ from that of $\sigma 3$ protein when the two proteins are coexpressed in transfected cells.

Amplification of pSVP1KIN Plasmids in Transfected Cells. The difference in P1 protein synthesis observed between COS cells transfected with wt as compared with mutant K296R P1 expression vector could potentially be caused by a difference at the level of pSVP1KIN plasmid DNA replication or transcription or at the level of P1 mRNA translation. Amplification of the wt and mutant K296R pSVP1KIN plasmid vectors in transfected COS cells was not detectably different as measured by Southern blot hybridization of Hirt supernatant DNA fractions digested with *EcoRI*. The amount of the predicted 829-base-pair (bp) P1-specific fragment was comparable for DNA isolated from wt- and mutant-transfected cells when cultured in the absence of 2-aminopurine (Fig. 5, lanes 3 and 5). The fragment was not detected in mock-transfected cells (lane 2). Treatment of cultures with 2-aminopurine beginning 3 hr after transfection drastically reduced the amplification of both the wt and the mutant P1 vector plasmids (lanes 4 and 9) as compared with parallel cultures not treated with 2-aminopurine (lanes 3 and 5).

Northern Gel-Blot Analysis of P1 mRNA Expression in Transfected Cells. Northern gel-blot analysis revealed that the level of P1 mRNA was slightly greater in cells transfected with the P1 K296R mutant as compared with the P1 wt vector (Fig. 6, lanes 2 and 3). The P1 KIN cDNA probe hybridized to two P1-specific mRNAs in transfected cells, the predicted

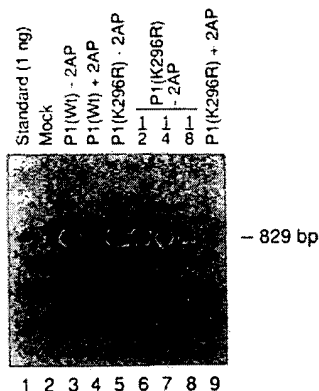


FIG. 5. Replication of wt and K296R mutant P1 plasmids in transfected COS cells. COS cells were transfected with the pSVP1KIN expression plasmids; 48 hr later Hirt extracts were prepared, and DNA was digested with *EcoRI* and then analyzed by Southern gel-blot hybridization using as the probe the 32 P-labeled 0.8-kb *EcoRI* fragment of the P1 KIN cDNA at 1×10^6 cpm/ml. Where indicated, cells were treated with 10 mM 2-aminopurine (2AP) beginning after chloroquine treatment. Lane 1 (Standard), 1 ng of pSVP1KIN(Wt) plasmid digested with *EcoRI*; lane 2 (Mock), DNA from mock-transfected COS cells; lanes 3 and 4 [P1(Wt) -/+ 2AP], DNA from wt pSVP1KIN(Wt)-transfected cells cultured in the absence (-) or presence (+) of 2AP; and lanes 5-9 [P1(K296R) -/+ 2AP], DNA from mutant pSVP1KIN(K296R)-transfected cells cultured in the absence (-) or presence (+) of 2AP. Lanes 6, 7, and 8 contained 1/2, 1/4, and 1/8, respectively, of the amount of DNA sample analyzed in the other lanes. The position of the 829-bp *EcoRI* fragment standard is indicated.

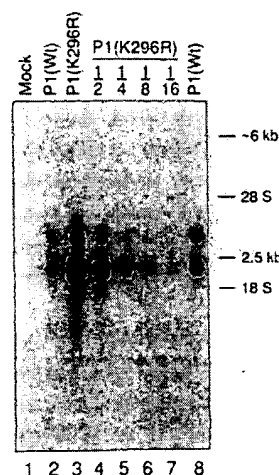


FIG. 6. Steady-state level of plasmid-derived P1 mRNA in transfected COS cells. COS cells were transfected with the pSVP1KIN expression plasmids; 48 hr later total cellular RNA was isolated (28) and analyzed by Northern gel-blot hybridization using the 1.8-kb *HindIII/PstI* fragment of the P1 KIN cDNA (11) as the probe. Lane 1 (Mock), RNA from mock-transfected COS cells; lanes 2 and 8 [P1(Wt)], RNA from wt pSVP1KIN(Wt)-transfected cells; and lanes 3-7 [P1(K296R)], RNA from mutant pSVP1KIN(K296R)-transfected cells. Lanes 4, 5, 6, and 7 contained 1/2, 1/4, 1/8, and 1/16, respectively, of the standard amount (5 μ g) of total RNA. The position of the 18S and 28S ribosomal RNAs and the 2.5-kb and ~6-kb P1 transcripts observed in human amnion U cells are indicated.

2.3-kb species which was the major P1-specific mRNA, and a minor P1 mRNA of about 2.8 kb. The minor mRNA is likely a splice-site variant of the SV40 leader specified by the pJC119 vector (29). Quantitation of dilution standards by laser densitometry revealed that the steady-state amount of mutant P1 mRNA was 3-fold greater than that of wt P1 mRNA in transfected cells (Fig. 6, lanes 2, 4, and 5).

DISCUSSION

In this paper we provide information regarding the successful expression, and the regulation, of human P1/eIF-2 α protein kinase cDNA clones in mammalian cells. Two important points emerge from the studies. First, using transient transfection assays, we demonstrated the synthesis of both the wt and a phosphotransfer mutant (K296R) P1 protein in transfected COS monkey cells. Second, the K296R mutant P1 protein was synthesized at a greatly elevated level in COS cells relative to the wt P1 protein, whereas in cell-free protein-synthesizing systems the wt and K296R mutant P1 mRNAs were translated with comparable efficiencies.

Approximately 30-fold more K296R mutant P1 protein was synthesized than wt P1 protein in COS cells transfected with pSVP1KIN cDNA expression plasmids. Systematic analyses revealed that the DNA template copy number was comparable for the K296R mutant and wt P1 plasmids and that the steady-state amount of the K296R mutant P1 mRNA was only about 3-fold greater than that of wt P1 mRNA. However, the amount of mutant K296R P1 protein was at least 30-fold greater than that of wt P1 protein. This difference in amount of P1 protein likely reflects a difference in rate of P1 protein synthesis, rather than degradation, because both pulse-labeling with [35 S]methionine and Western immunoblot analysis revealed a comparably enhanced level of P1 protein in mutant- as compared with wt-transfected cells.

Whereas a part of the increased amount of mutant P1 protein may be attributed to the 3-fold higher transcript level,

most of the 30-fold difference between wt and mutant P1 protein levels is due to an increased translational efficiency of the mutant P1 mRNA. No difference in replication of K296R mutant and wt plasmid DNA vectors was observed. The differential expression of the K296R mutant P1 protein over that of the wt P1 protein is not limited to plasmid-derived P1 mRNAs expressed from the SV40 late promoter-enhancer in COS cells containing the pJC119 vector as described herein. Similar results have been obtained with the baculovirus vector pEVmXIV and Sf21 insect cells (30); baculovirus recombinants are readily generated that efficiently express the K296R mutant P1 protein but not the wt P1 protein (D.C.T. and C.E.S., unpublished observations).

These observations regarding the differential efficiency of expression of wt and K296R mutant P1 kinase may be related to the effects of IFN on the expression of plasmid-derived mRNAs in COS cells. IFN treatment inhibits the expression of vesicular stomatitis virus *G* gene and reovirus *S3* gene in pSVG- and pSVS3-transfected COS cells at the level of translation (29, 31). Furthermore, disruption of the RNA-dependent P1 kinase pathway—for example, by expression of kinase antagonists such as adenovirus VA RNA or eIF-2 α subunit mutants (S51A) or homologues (K3L)—increases the translational efficiency of plasmid-derived mRNAs in COS cells (32, 33).

The reason why the K296R point mutant, which lacks P1 kinase activity, would give about a 3-fold higher P1 transcript level in transfected cells than the wt P1 kinase is unclear, but it may indicate a role for the P1 kinase in the modification of a transcriptional component utilized by the SV40 late promoter-enhancer. Consistent with this possibility, the expression of adenovirus VAI RNA, an antagonist of the P1 kinase (2), in monkey CV1p cells causes a 3- to 4-fold increase in the synthesis of SV40 tumor (T) antigen mRNA (34).

Autoregulation of gene expression is an important mechanism in mammalian cells. Although autoregulation was first clearly demonstrated at the transcriptional level, as exemplified by SV40 T antigen expression (35), the mRNA translation level is also a step for control of expression by autoregulation. The synthesis of the iron binding and storage protein ferritin, for example, is primarily regulated at the translational level (36). The apparent autoregulation of P1 expression is dependent upon P1 kinase catalytic activity and not simply P1 RNA-binding activity. Both wt P1 and mutant K296R P1 possess RNA-binding activity but only wt P1, which was autoregulated, is an active kinase (D.C.T., S. J. McCormack, J. P. Doohan, and C.E.S., unpublished observations, and refs. 13–15).

It is well established that the RNA-dependent P1 kinase (PKR) can selectively bind to, and be regulated by, certain structured single-stranded RNAs (2). Conceivably the RNA-dependent P1 protein kinase binds to and is activated by its own mRNA, thereby autoregulating P1 protein synthesis at the level of translation. This autoregulation of a key enzyme of the IFN-induced antiviral response (2, 10) could be especially important during the period of host recovery from virus infection, when perhaps higher levels of P1 protein are no longer beneficial to the cell.

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An embryonic DNA-binding protein specific for a region of the human IFN β_1 promoter

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ABSTRACT

Embryonal carcinoma (EC) cells are unable to make interferon in response to inducing agents. This block disappears after differentiation. We have found that nuclear extracts from undifferentiated P19 EC cells contain a DNA-binding activity which specifically recognizes a region within the human interferon- β_1 promoter. This activity is absent from differentiated cell types, both of EC and non-EC origin. The binding of the factor in undifferentiated EC cells leads to dramatic changes in the overall protein binding pattern of the interferon promoter as compared with differentiated cells, and may be responsible for repression of the endogenous interferon- β gene prior to differentiation.

INTRODUCTION

Embryonal carcinoma (EC) cells, the stem cells of the teratocarcinomas, have been used extensively as an *in vitro* system to study mammalian development and cell differentiation. The rationale for their use is the extensive similarity between these cells and those of the early embryo (1). The resemblance includes morphology, expression of a number of markers, and above all, pluripotentiality. EC cells will differentiate into a variety of tissue types both in culture and in tumours (1). Furthermore, some EC cell lines have been shown to be capable of contributing to all tissues in chimeric mice (1), thus demonstrating their functional totipotency.

A number of positive and negative markers for distinguishing EC cells from their differentiated derivatives have been described (1). These include a number of cDNA clones corresponding to mRNAs whose expression either increases or decreases during differentiation (2,3). To date little is known about the molecular mechanisms involved in the regulation of expression of these genes beyond identification of transcriptional and post-transcriptional components. The replication of a number of virus types is blocked or inefficient in EC cells (4,5,6). Although this appears to be a complex phenomenon, one component is certainly the decreased activity of the viral promoters in

the undifferentiated cells. In particular the enhancers of papovaviruses such as polyoma and SV40 and of some retroviruses seem to be inactive in EC cells. Evidence has been put forward for this being due to negative regulation in the undifferentiated cells and in some cases EC cell specific DNA-binding factors which recognize viral enhancer elements have been identified (7).

One of the negative markers for the undifferentiated state of EC cells is their inability to produce interferon (IFN) in response to exposure to viruses or double stranded RNA (8,9), a characteristic shared with early embryos (10). The IFNs are potent biological agents which exert pleiotropic effects on target cells or organisms (11). All of the IFN genes are strictly inducible with no expression being detected in untreated cells (11). Following exposure to inducers, transcriptional activation of the appropriate IFN gene or genes occurs. All differentiated cell types are competent for IFN induction. This also applies to both derivatives of EC cells and to differentiated cells of early embryos. Thus during cell differentiation a transition occurs from a state where IFN genes are incompetent to one where they can respond to induction (8,10). We have asked the question of whether or not differences could be found in the spectrum of DNA-binding proteins specific for the regulatory regions of an IFN gene when nuclear extracts from undifferentiated and differentiated cells were compared. We report that nuclear extracts from undifferentiated EC cells contain such a DNA-binding factor which is absent from differentiated cell types of both EC and non-EC origin. The binding of this factor to the HuIFN β promoter in EC cells leads to dramatic changes in the overall protein binding pattern of the region, as compared with differentiated cells, and may be responsible for repression of endogenous IFN expression prior to differentiation.

MATERIALS AND METHODS

Cells and plasmids

pIFN β -Hu was prepared by cloning a 1.8 kb EcoRI fragment with the coding and upstream sequences of HuIFN β , into the polylinker of pGEM-1 (Promega Biotec, Madison, WI, USA). pHuIFNPr was prepared from this plasmid by subcloning the 282 bp EcoRI-HincII fragment containing the 5' upstream sequences of the gene into the polylinker of pGEM-1. Plasmids containing the DraI-AluI, AluI-AvaII, and DraI-AvaII fragments of this 5' region were a gift of John Hiscott.

Murine P19 EC cells were maintained as described previously (12) and differentiated with retinoic acid or DMSO according to the method of Rudnicki and McBurney (13).

Induction and titration of IFN production

Levels of IFN production were determined as described previously (14). For induction cells were exposed to Newcastle Disease Virus (NDV) (approximately 100 PFU/cell) for two hours. The virus-containing medium was removed

and fresh medium was added. The next day aliquots of this conditioned medium were removed, spun to remove cell debris, and added to cells in microtiter plates. The presence of HuIFN in the medium was detected by challenging the T98G cells with serial dilutions of Encephalomyocarditis (EMC) virus as described (15). MuIFN levels were determined using L929 cells challenged with vesicular stomatitis virus (VSV) (16).

Preparation of nuclear extracts

Nuclear extracts were prepared according to the method of Dignam *et al.* (17) from L cells, undifferentiated P19 cells, and P19 cells differentiated with DMSO. The extracts were aliquoted and stored in liquid nitrogen. Protein determinations were done using the Bio-Rad Protein assay kit (BioRad, Mississauga, Ont., Canada).

Gel retardation assay

A fragment of the 5' non-coding region of the HuIFN β_1 gene was end-labelled with 32 P-ATP (18) and mixed with 10 μ g of nuclear extract in the presence of 250 ng HinfI digested pBR322, 6 μ g poly (dI-dC)-poly (dI-dC), 1 mM DTT, 25 mM Hepes, pH 7.9, 40 mM NaCl, 1 mM EDTA, and 5% glycerol (for a total volume of 25 μ l). pGEM-1 contains a HindIII site in the polylinker adjacent to the HincII site used for cloning, and in some cases this alternate site was used in preparation of the end-labelled fragments. For certain experiments synthetic oligonucleotides (prepared by G. Boileau, Université de Montréal) or subfragments of the promoter region were included to serve as specific competitor DNAs. The reaction mixture was incubated for 25 minutes at 25°C, following which 3 μ l blue II sample buffer (18) was added and the samples were electrophoresed on 5% polyacrylamide TGE gels (50 mM Tris, pH 7.5, 380 mM glycine, 2 mM EDTA). After the samples had migrated sufficiently the gels were dried and autoradiographed.

DNase footprinting

Nuclear extracts were incubated with end-labelled fragments as for the gel retardation experiments. At the end of the 25 minute incubation period digestion with DNase I was performed according to the method of Jones *et al.* (19), using 50 ng DNase for samples without nuclear extract and 100 ng for samples with extract. The samples were extracted with phenol/chloroform, ethanol precipitated, and run on 5 or 8% sequencing gels. Lanes containing Maxam-Gilbert G+A reactions were included on each gel for calibration purposes but are not shown in the figures.

RESULTS

Expression of the human IFN β_1 gene in P19 cells.

Since genomic clones of the murine IFN β (MuIFN β) gene were not available to us at the time we started this work, we first ascertained whether or not the heterologous HuIFN β_1 gene was regulated appropriately in murine EC cells. A 1.8 kb EcoRI genomic fragment containing 282 bp of upstream and 713 bp of downstream coding sequence (20) was subcloned into pGEM-1, then co-transfected into P19 EC cells with the vector pSVtkneo β , which encodes resistance to the drug G418. A pool of G418 resistant colonies (approximately 250) was obtained and the ability of these cells to produce human and murine IFN in response to NDV was tested prior to and following differentiation. The production of human IFN β_1 (HuIFN β_1) is distinguished from murine IFNs by testing the protective effect of the media from induced cells on human cell lines, since

Transfected	Differentiated	Induced	HuIFN titer	MuIFN titer
-	-	-	ND	<6 IU
-	-	+	ND	<6
-	+	-	ND	<6
-	+	+	ND	57600
+	-	-	<6 IU	<6 IU
+	-	+	<6	870
+	+	-	<6	<6
+	+	+	150	57600

Table 1. Titration of HuIFN β and MuIFN produced by transfected P19 cells. P19 EC cells were transfected with the 1.8 kB EcoRI fragment containing the sequence for HuIFN- β , (including 282 bp of 5' upstream sequence and the entire coding sequence). The spent culture media from the cells (either undifferentiated or differentiated with retinoic acid) were analysed for the presence of IFN before and after induction by NDV. The presence of HuIFN in the media was measured by challenging T98G cells with EMC virus while MuIFN levels were tested by challenging L929 cells with VSV. ND, not done.

IFNs cross-react poorly between species (11). As can be clearly seen from the results presented in Table 1, no HuIFN expression was found in the undifferentiated EC cell population either before or after induction. Following differentiation a significant titer of HuIFN was detected only in media from the virus-treated cells. Differentiated cultures derived from transfected or control non-transfected populations produced high levels of MuIFN when treated with virus. A relatively low, but significant, titer of MuIFN was observed in the virus-treated undifferentiated cultures of the transfected population, but never in non-transfected cells. Possible explanations of this will be discussed below. However, we conclude that the exogenous human gene is controlled by the regulatory factors responsible for repression of expression of the endogenous MuIFN gene in EC cells and that the transfected EcoRI fragment contains all the information necessary for this regulation.

Nuclear factors which bind to the HuIFN β promoter region

Figure 1 shows the restriction map and a schematic representation of the 5'-upstream region of the HuIFN β gene, with the location of the putative inducible enhancer indicated (21). This EcoRI-HincII fragment was subcloned into pGEM-1 (forming pHuIFNPr) in order to facilitate the preparation of end-labelled fragments which were used to analyse the interactions of nuclear factors with this part of the gene. In order to determine whether DNA-binding factors specific for the IFN gene could be detected in nuclear extracts we carried out gel retardation experiments with subfragments of the cloned 5'-upstream region. EcoRI-DraI, EcoRI-AvaII, AluI-HindIII and HaeIII-HindIII

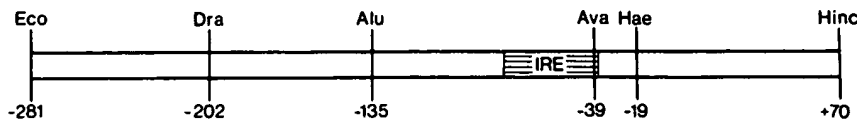


Figure 1. Schematic diagram of the 5' non-coding region of HuIFN β_1 . The numbering system of Zinn *et al.* (27) is used and indicates position relative to the mRNA cap site. IRE = Interferon Regulatory Element (22). Eco = EcoRI, Dra = DraI, Ava = AvaII, Alu = AluI, Hae = HaeIII, Hinc = HincII. The HincII site is immediately 5' to the ATG translation start site. The HindIII site in the pGEM-1 polylinker is 15 bp 3' of the HincII insertion site.

fragments were each tested for reactivity with nuclear extracts from L cells, which are highly competent for induction of their endogenous IFN genes (the HindIII site is only 15 nucleotides away from the HincII site in the pGEM-1 polylinker, thus digestion with either HindIII or HincII gives virtually identical restriction fragments). The EcoRI-AvaII and AluI-HindIII fragments both demonstrated a strong band of reduced mobility (Figure 2, lanes 4 and 7). These two fragments contain an overlapping sequence (corresponding to positions -135 to -39) which contains elements previously implicated in transcriptional control of the IFN β gene (21,23). The shifted bands could be competed



Figure 2. Nuclear factors interacting with subregions of the 5' untranslated region of HuIFN β_1 . The EcoRI-DraI (lanes 1,2), EcoRI-AvaII (lanes 3-5), AluI-HindIII (lanes 6,7) and HaeIII-HindIII (lanes 8,9) fragments were each end-labelled and tested for reactivity with nuclear extracts from L929 cells, as described in Materials and Methods. Lane 5 also contained unlabelled EcoRI-HindIII fragment (50 μ g) added as a specific competitor. Bands were visualized by autoradiography after electrophoresis of the samples through a low ionic strength gel.

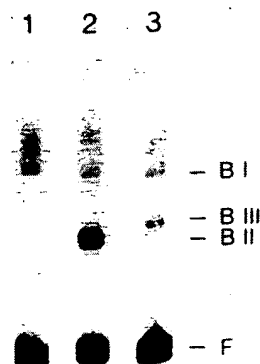


Figure 3. Nuclear factors interacting with the 5' non-coding region of HuIFN β_1 . Nuclear extracts (10 μ g) prepared from L929 cells (lane 1), undifferentiated P19 cells (lane 2), or P19 cells differentiated with DMSO (lane 3) were incubated with the end-labelled EcoRI-AvaII fragment. B I, B II, and B III indicate retarded bands I, II, and II, respectively; F indicates the unbound fragment.

by the addition of unlabelled EcoRI-HindIII fragment (Figure 2, lane 5). Neither the EcoRI-DraI nor HaeIII-HindIII fragments interacted sufficiently with the nuclear extracts to produce shifted bands. Subsequent experiments were performed using only the EcoRI-AvaII fragment (-281 to -39 bp upstream of the mRNA cap site).

Figure 3 illustrates the different interactions between the EcoRI-AvaII fragment and nuclear extracts prepared from L929 cells, P19 cells, or differentiated derivatives of the latter. Extracts from all three cell types lead to the appearance of a retarded band which migrates with the same relative mobility near the top of the gel (band I) (bands with common mobility are assigned the same band number without implying that the same factors are bound to the probe in each case). In lane 2, where the probe was reacted with an extract from P19 cells, a strong band of faster mobility (band II) was also seen. Band II is not detected with extracts from the nuclei of L929 cells (lane 1), 3T3 fibroblasts (not shown) or differentiated P19 cells (lane 3). However, with nuclear extracts from the latter a different band of somewhat lower mobility (band III) is obtained. Band III is usually less intense than

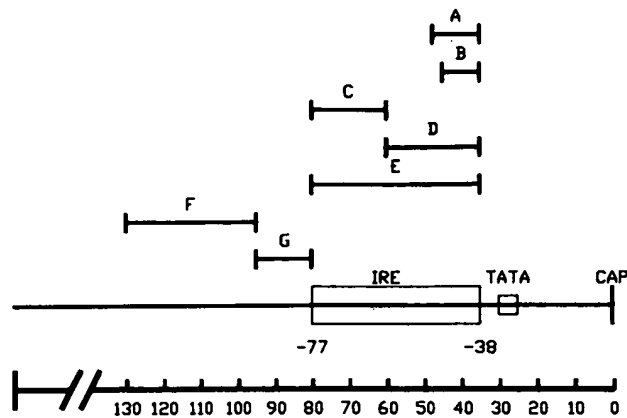


Figure 4. Relative positions of synthetic oligonucleotides used as competitors in gel retardation and DNase footprinting assays. The location of each of the oligonucleotides relative to the mRNA cap site is as follows: A (-47 to -35), B (-45 to -36), C (-79 to -64), D (-61 to -36), E (-79 to -36), F (-129 to -93) and G (-94 to -77). The locations of the cap site, TATA box, and IRE are also indicated.

band II, but this was found to be somewhat variable. The net result of this experiment is to show that a nuclear factor which specifically recognizes the HuIFNβ₁ promoter is present in EC cells (band II) and absent from fibroblasts and differentiated derivatives of EC cells. We have designated this factor as ECIF-1. Differentiated EC cells in turn contain a different DNA binding factor (band III).

A series of synthetic oligonucleotides derived from the HuIFNβ₁ upstream sequence were used as specific competitors in the gel retardation assay to further localize the interaction(s) of nuclear factors with the EcoRI-AvaII fragment. The relative positions of the oligonucleotides, which span the region from -129 to -38, are illustrated schematically in Figure 4. The shifted bands from all three extracts could be competed most efficiently with oligonucleotide F, corresponding to the sequence between -129 and -93. Results for extracts from undifferentiated and differentiated P19 cells are shown in Figure 5. In addition, the bands from extracts of undifferentiated P19 cells showed partial competition by oligonucleotide G while the bands from the differentiated P19 extracts were slightly inhibited by oligonucleotide E (-79 to -36), the region identified by Goodbourn *et al.* (21) as corresponding to the inducible enhancer of the HuIFNβ₁ gene (IRE).

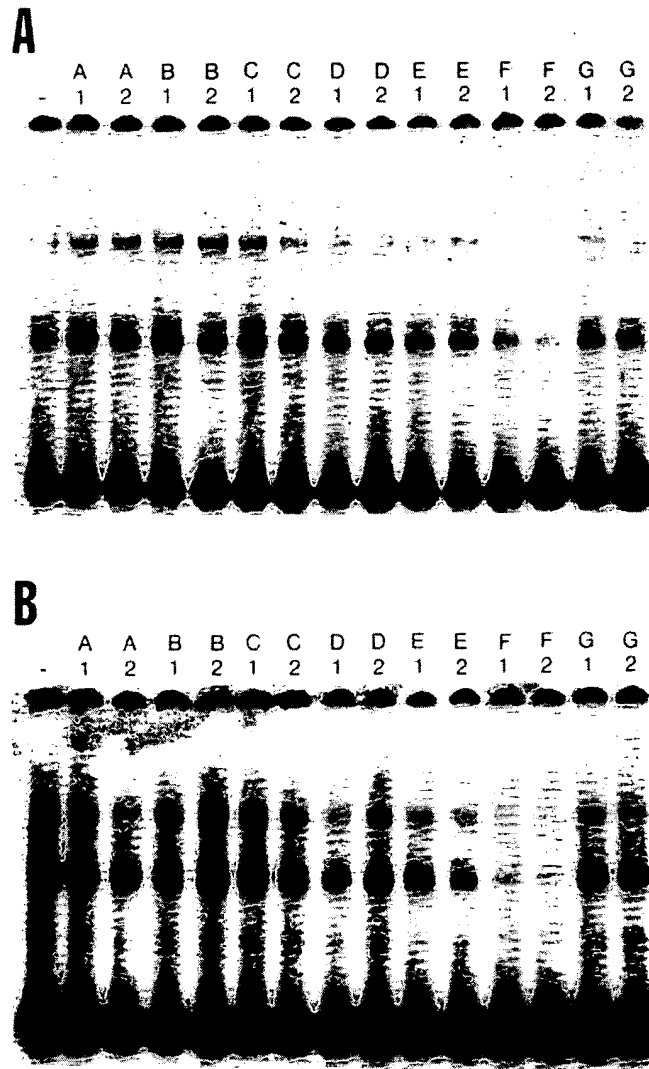


Figure 5. Gel retardation assay with P19 nuclear extracts showing competition by synthetic oligonucleotides. Nuclear extracts from undifferentiated (A) or DMSO-differentiated (B) P19 cells were incubated with the end-labelled EcoRI-AvaII fragment in the absence (-) or presence of oligonucleotides A through G as indicated above each lane. Either 100 or 200 ng of each competing oligonucleotide was added as specified by lanes marked 1 or 2 respectively.

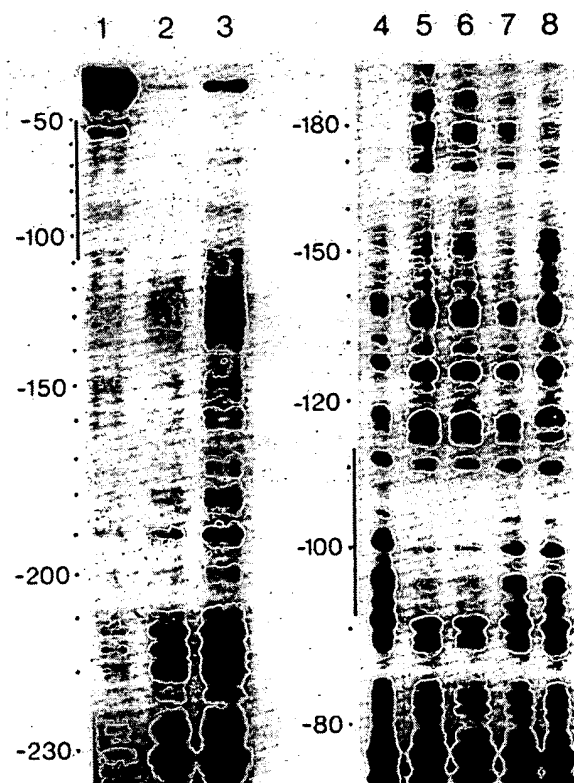


Figure 6. DNase footprinting assay with nuclear extracts from undifferentiated P19 cells. The EcoRI-AvaII fragment (labelled at either the 5' or 3' end) was incubated with nuclear extracts (20 μ g) from undifferentiated P19 cells, subjected to DNase digestion, and electrophoresed on a sequencing gel. Lanes 1-3 show the DNase digestion pattern of the coding strand in the absence (lane 1) or presence of extract (lanes 2 and 3) and oligonucleotide F (lane 3). Lanes 4-8 show the noncoding strand digested with DNase in the absence (lane 4) or presence (lanes 5-8) of extract and fragments DraI-AluI, AluI-AvaII, and DraI-AvaII (lanes 6, 7, and 8 respectively). Numbers beside each gel mark the position relative to the mRNA cap site. Protected areas are indicated by a bar.

DNase footprinting of the protein/DNA interactions

In order to better define the regions which bind factors from the three nuclear extracts under investigation, DNase I footprinting experiments were performed. The EcoRI-AvaII fragment was labelled at either the 5' or 3' end to enable detection of interactions on the coding or non-coding strands re-

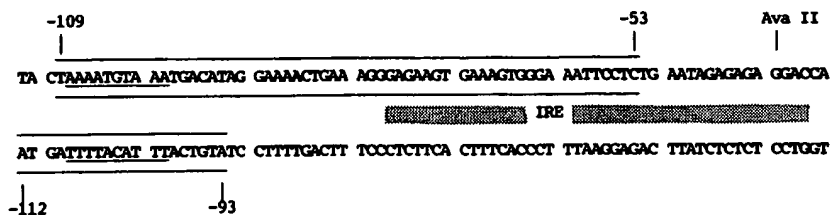


Figure 7. Schematic diagram of the footprint found with undifferentiated P19 cells. The protected regions on the coding and non-coding strands are enclosed by bars and the endpoints are indicated. The sequence within the footprint with homology to the Ela promoter is underlined. The IRE and the AvaII restriction site are also marked.

spectively. With nuclear extracts from undifferentiated P19 cells a protected region is observed which spans from -109 to about -53 on the coding strand and from -112 to -93 on the non-coding strand. Presumably this is due to binding of ECIF-1. The gels are seen in Figure 6 while the region of protection is illustrated schematically in Figure 7. The endpoint of the coding strand footprint is difficult to ascertain with accuracy since the resolution of the sequence decreases as one approaches the end of the fragment. Oligonucleotide F, which overlaps most of the region of the coding strand footprint and all of the non-coding strand footprint, can compete away the binding protein(s) (Figure 6, lane 3) as can the AluI-AvaII and DraI-AvaII fragments which also cover the region of the footprint (Figure 6, lanes 7 and 8). The DraI-AluI fragment, on the other hand, does not compete (Figure 6, lane 6).

In view of the footprints obtained with extracts from undifferentiated P19 cells two additional synthetic oligonucleotides were tested for their ability to compete the DNA-protein interactions. Oligonucleotide J corresponds to the region protected on the non-coding strand (*i.e.* -112 to -93) while oligonucleotide H corresponds to a shorter sequence (-108 to -99, underlined in Figure 7). Oligonucleotide J exhibited good competition of retarded bands in gel retardation experiments with nuclear extracts from both undifferentiated and differentiated P19 cells (Figure 8 and results not shown) while oligonucleotide H, shorter by only 10 base pairs (four from the 5' end and six from the 3' end), did not compete at all.

The footprints observed with nuclear extracts from differentiated cells (either L cells or DMSO-treated P19 cells) (Figure 9) are strikingly different from those observed with extracts from undifferentiated P19 cells. A large



Figure 8. Gel retardation assay with nuclear extracts from undifferentiated P19. Nuclear extracts from undifferentiated P19 cells were incubated with the end-labelled EcoRI-AvaII fragment in the absence (lane 1) or presence of oligonucleotide H (lane 2) or J (lane 3). Oligonucleotide H comprises the sequence -108 to -99 while oligonucleotide J spans -112 to -93.

region of interaction on both the coding and non-coding strands was found with nuclear extracts from these cells, stretching from approximately -210 to -70 (as mentioned above the exact endpoints of the footprint cannot be accurately determined due to difficulty with resolution of the sequence at the ends of the fragment). The entire footprint can be competed by the DraI-AluI, AluI-AvaII, and DraI-AvaII fragments (Figure 9) as well as by oligonucleotide F (Figure 10). The most efficient competitors, DraI-AluI and oligonucleotide F, cover only a small portion of the protected sequence. This suggests that the cooperative binding of more than one protein is occurring in these differentiated cells.

DISCUSSION

The induction of the IFN genes has been shown by several groups to involve cis-acting sequence elements located within the first several hundred base pairs of the capping site and in all probability trans-acting proteins which interact with them. Both positive and negative regulation have been described, and an inducible enhancer has been located between -77 and -37 of the HuIFN β_1 gene (21). This same group also used in vivo DNase footprinting to show the existence of two regions within the HuIFN β_1 gene promoter which bind protein (24).

In addition to the "on/off" regulation of induction the type I IFN genes are susceptible to other forms of regulation. These include genetic regulation of the levels of IFN transcription obtained following induction (25,26) and a developmental regulation which leads to undifferentiated cell types being incompetent for IFN induction (8,9). In the first part of this publica-

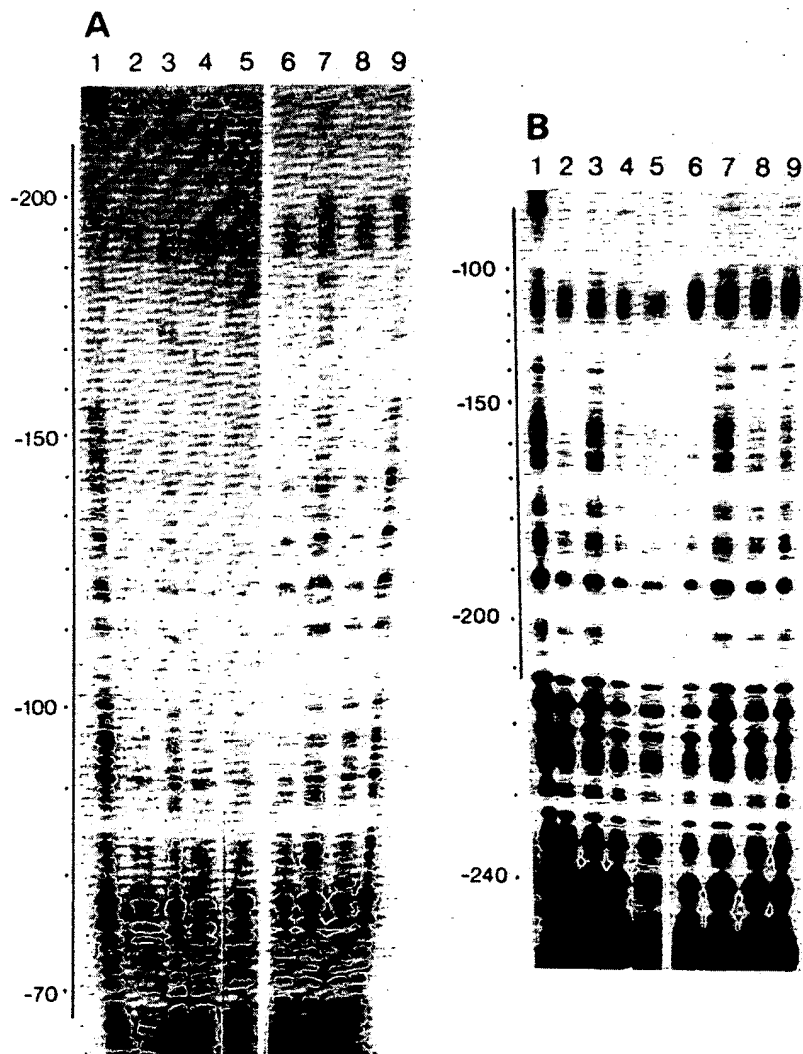


Figure 9. DNase footprinting assay with nuclear extracts from differentiated cells. The EcoRI-AvaII fragment (labelled at either the 5' or 3' end) was incubated with nuclear extracts from differentiated cells, subjected to DNase digestion, and electrophoresed on a sequencing gel. (A) Lanes 1-9 show the noncoding strand digested with DNase in the absence (lane 1) or presence of 20 μ g of extract from L929 cells (lanes 2-5) or differentiated P19 cells (lanes 6-9). Competitions were performed using 400 ng of fragments DraI-AluI (lanes 3 and 7), AluI-AvaII (lanes 4 and 8), or DraI-AvaII (lanes 5 and 9). (B) Lanes 1-9 show the pattern of protection on the coding strand. Each lane contains the same extracts and competitors as in (A). Numbers beside each gel mark the position relative to the mRNA cap site. Protected areas are indicated by a bar.

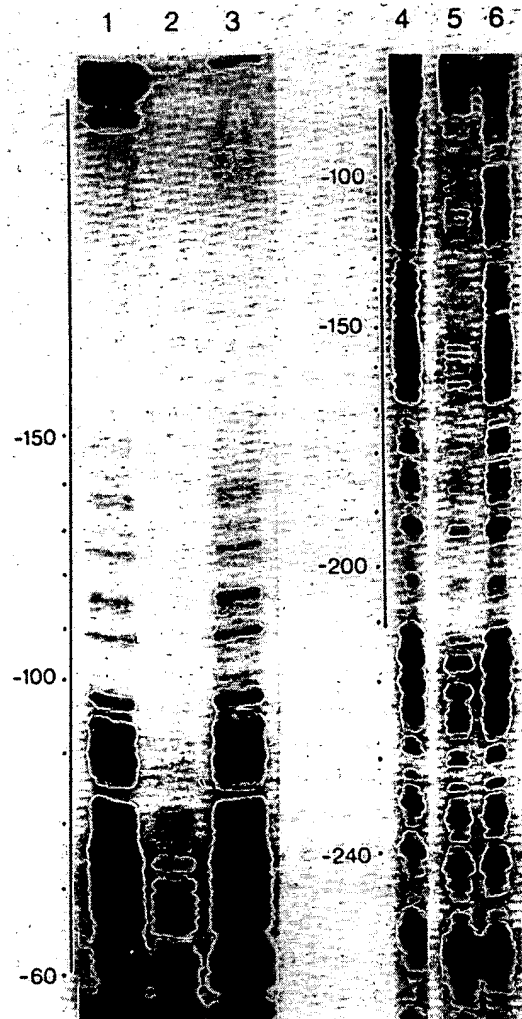


Figure 10. Inhibition of footprints by oligonucleotide F. The EcoRI-AvaII fragment was incubated with nuclear extracts from differentiated cells, subjected to DNase digestion, and electrophoresed on a sequencing gel. Lanes 1-3 show the DNase digestion pattern of the non-coding strand in the absence (lane 1) or presence of L929 nuclear extract (lanes 2 and 3) and oligonucleotide F (lane 3). Lanes 4-6 show the noncoding strand digested with DNase in the absence (lane 4) or presence (lanes 5-6) of nuclear extracts from differentiated P19 cells and oligonucleotide F (lane 6).

tion we have shown that the H1FN β , gene with 282 bp of upstream sequence, i.e. with all of the cis-acting elements implicated in the induction of the gene, shows the appropriate pattern of expression in murine EC cells (Table

1). Undifferentiated cells do not express the gene even following induction, whereas the gene is inducible following differentiation of the EC cells. Thus the region of the gene which was transfected carries all of the necessary information for determining the competence of the gene. Similar regulation of gene expression across species barriers was observed in several differentiated cell lines when human IFN α was transfected into mouse L cells (27) or when HuIFN β_1 was transfected into mouse FM3A cells (20) or C127 cells (28).

In the transfection experiments we noted that a low level of endogenous MuIFN was detected when undifferentiated populations transfected with the HuIFN β_1 gene were infected with NDV. This could be due to a low level of spontaneous differentiation of the cells following the manipulations involved in the transfection. Alternatively, the presence of the heterologous gene may have "titred out" a negative regulatory factor involved in repression of IFN gene expression in EC cells.

Gel retardation experiments with nuclear extracts from L cells, undifferentiated P19 cells, and P19 cells differentiated with DMSO, demonstrate that significant differences exist between these cells types with regard to their interactions with the 5' upstream sequences of the HuIFN β_1 gene (Figure 3). All three extracts exhibit a band of common mobility (band I), although the gel retardation experiments are insufficient to determine whether this is in fact a common factor or different factors forming DNA-protein complexes which migrate with the same mobility. Both untreated and differentiated P19 cells were found to contain an additional factor which led to the appearance of a band of greater mobility in the gel retardation assay. However the complex obtained in EC cells (band II, ECIF-1) differed from that seen with nuclear extracts from differentiated derivatives (band III) by mobility, (and in some cases was more intense than the latter). Since these bands were of differing mobility it is likely that they are due to the interaction of different factors from the two extracts with the EcoRI-AvaII probe. The differences seen between L cells and DMSO-treated P19 cells in the gel retardation experiments may be partly due to the fact that they are different types of cells. The localization of regulatory sequences by other methods has been seen to depend, at least in part, on the type of cell under investigation (21,23,28,29).

Competition of gel retardation bands with synthetic oligonucleotides spanning the HuIFN β_1 promoter region showed oligonucleotide F (corresponding to positions -129 to -93) to be the most efficient competitor (Figure 5). This region contains several interesting features: a region (-167 to -94)

shown by Zinn and Maniatis (24) to bind protein *in vivo* and a region which presents a high degree of sequence homology to part of the HuIFN α_1 (30) and IFN α_2 (21) promoters. This region also contains several of the hexamer repeats described by Fujita *et al.* (23) as important in obtaining full induction of the gene.

Further details of the DNA/protein interactions detected in the gel retardation experiments were obtained by DNase footprinting. Extracts of undifferentiated P19 nuclei protected a relatively short region within the sequence of oligonucleotide F (Figure 6). The extent of the footprint is longer on the coding strand, reaching into the IRE, whereas it ends upstream of the IRE on the non-coding strand. In spite of the asymmetric nature of the interaction, oligonucleotide F completely inhibits the interaction, although it contains no sequence from the extreme downstream part of the footprint. Furthermore, an oligonucleotide corresponding to the sequence which spans from -109 to -93, the part of the footprint which is protected on both strands, can alone compete efficiently for the interaction in both gel retardation and footprinting experiments. A shorter version of this sequence containing only the "box I" sequences (positions -108 to -99) is a much less efficient competitor: although it can compete in the footprint reaction (data not shown) it does not compete the gel retardation bands (Figure 8).

The DNase footprinting pattern obtained with extracts from the nuclei of L cells or differentiated P19 cells is very different from that with the P19 extracts (Figure 9). The footprint covers a long region spanning from at least -210 to -70 and containing the region protected by extracts of undifferentiated cells. Some interruption of the footprint can be observed if the footprints are examined closely, indicating that multiple proteins may be involved. The patterns of the two types of differentiated cells are very similar. In both cases the whole footprint is competed out by oligonucleotide F (Figure 10), which represents only a small part of the total protected region. We interpret this to mean that cooperativity of binding exists, with occupancy of a binding site upstream of the IRE being obligatory for the binding of other factors. Other workers have provided evidence in related systems for functional cooperativity in this region. For example, Fujita *et al.* (23) have found that multiple copies of a hexamer repeat (homologous to the sequence AAATGT contained within this footprint) are necessary to confer inducibility on a reporter gene. Dinter and Hauser (31) found that duplication of the sequence from -90 to -51 resulted in increased IFN β_1 expression after virus exposure. Kuhl *et al.* (30), using a similar repeated sequence found in the

IFN α 1 promoter, found that multimers of the repeat are needed for inducibility and that monomers and dimers were insufficient.

The 5' end of the footprint (protected on both strands) also exhibits homology to a viral regulatory region: the sequence (GGAAGTGAAA) is partially homologous (5/10 bases the same) to the Ela enhancer core (32). Downstream, in the region protected only on the coding strand, is another sequence with even greater homology to the Ela enhancer core sequence (9/10 bases the same). The homology may be significant because it has also been shown that EC cells contain an activity which can replace the requirement for the adenovirus Ela product in replication of this virus (33,34). This activity, considered by some authors to be responsible for an enhancer-repressing activity (34), is lost upon differentiation.

It is tempting to speculate that ECIF-1 is a negative regulatory factor which binds to the IFN promoter in undifferentiated EC cells, preventing attachment of the alternate factors which bind here after differentiation. In the presence of ECIF-1 the gene is not capable of responding to induction. Several lines of evidence support this idea. The footprinting experiments show clearly that in undifferentiated cells only this site is occupied, as opposed to multiple sites in differentiated cells (a specific DNA-binding protein from undifferentiated EC cells has similarly been demonstrated in a retrovirus system (5)). Furthermore, we have found that cell fusions between EC cells and differentiated cells which normally are competent for IFN expression behave like the EC parent, and become inducible only if made to differentiate (unpublished results). It has also been shown that an enhancer-repressing (or Ela-like) activity exists in EC cells and that this activity disappears upon differentiation (33) supporting the involvement of a negative factor in the EC cell phenotype. One last curious point is that the sequence AAATGTAAA in the central region of the footprint is highly homologous to the sequence TAAATATAAAA, a functional part of a transcription silencer in yeast (35). In light of the increasing evidence for interchangeability of yeast transcription factors and those of higher eukaryotes (36,37), the similar sequences may indicate homology of function.

It is important to note that the results of gel retardation and footprinting experiments do not always coincide completely. The most notable example is seen where footprints using extracts from L cells or from differentiated P19 cells were almost identical, whereas in gel retardation experiments the latter extracts yield an additional band. It was also noted that certain oligonucleotides which did not compete in gel retardation did compete in the

footprint reaction. The relationship of the complexes observed in the gel retardation experiments to those giving rise to footprints is not entirely clear. Differences in gel retardation and footprint results are probably due to the different demands put on the stability of the complexes in the two techniques.

It is not possible at this time to deduce the relationship between the protein factors observed here and those detected by others or to determine the role of the varied protein binding regions observed in differentiated cells. Both positive and negative factors have been implicated in the induction of the HuIFN β gene in cells competent for IFN production (38,39,40). Our results suggest that when cells which are not competent for IFN production acquire the ability to be induced they undergo a concurrent alteration in the factors controlling the regulation of the IFN gene. At this point we do not wish to speculate on the possibility of changes in the pattern of factors following induction.

In conclusion we report here the existence of a DNA-binding factor in nuclear extracts of P19 EC cells which recognizes a specific sequence within the promoter region of the HuIFN β gene. This activity is absent in differentiated cells of both EC and non-EC origin. The factor, which we have called ECIF-1, shows some of the properties to be expected of a tissue specific repressor of transcription. Work is in progress to determine the regulatory nature of this protein and the *cis*-acting activity of its binding site.

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CHANGES IN THE CONFORMATION OF THE INTERFERON β GENE DURING
DIFFERENTIATION AND INDUCTION

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SUMMARY: DNase I sensitivity was used to investigate the chromatin conformation of the interferon β gene during differentiation of the mouse teratocarcinoma cell line PC13. These cells do not produce interferon upon viral induction in their undifferentiated state, but do so on differentiation from stem cells to endoderm. Only in induced differentiated cells were the interferon β genes digested by DNase I. A similar effect was seen in a line of human cells (MG63) upon induction. We conclude that it is induction of interferon production that brings about the change in the DNase I sensitivity of these genes, rather than differentiation.

In the last few years the ability of DNase I to distinguish between the chromatin conformation of "active" and "inactive" genes has been well documented (1,2,3,4). Most of the research has centred upon the comparison of a particular gene in different tissues and has shown that for most genes only those being transcribed in that tissue are DNase I sensitive. Studies using erythroleukemic cell lines (5) have shown that the globin genes that are capable of being transcribed upon induction, are also DNase I sensitive, suggesting that the change in chromatin configuration of a gene detected with DNase I is related to the commitment of the cell to a particular developmental pathway, and the use of that gene, rather than its actual transcriptional state. However, this does not hold true in all systems studied, for example certain muscle specific genes alter their DNase I sensitivity during myogenesis (6) as do some of the *Drosophila* heat shock protein genes upon a

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temperature shock (7,8), and under these conditions, transcription of the DNase I sensitive genes occurs.

The interferon (IFN) genes provide a convenient mammalian system for study of an inducible gene. Prior to induction of these cells with virus, no IFN protein or IFN mRNA is produced, whilst a few hours after the addition of virus both IFN mRNA and protein can be detected. Further, teratocarcinoma cells provide a suitable system to investigate the control of the interferon system during differentiation, since only differentiated cells are capable of induction (9). We therefore studied DNase I sensitivity of the IFN β gene in these cells before and after differentiation, and before and after induction. We also examined a differentiated human cell line (MG63) before and after induction.

MATERIALS AND METHODS

Cell culture. Non-differentiated PC13 cells were grown in modified Eagles medium plus 7.5% fetal calf serum, 2.5% newborn calf serum and antibiotics (10) on gelatinized plastic flasks. The cells were differentiated by the addition of 3 μ g/ml retinoic acid and grown in non-gelatinized flasks. After 10 days the cells are capable of producing IFN upon induction (9). Induction by NDV (Strain F: 2500 HAU/ml) or Sendai virus (12 HAU/ml) was for 1 hour in 2% serum media, followed by a further virus-free incubation of 3 hours. This was found to be the time when IFN could first be detected in the supernatant of differentiated cells (data not shown). MG63 cells were grown in Glasgow modified Eagles media plus 10% newborn calf serum and antibiotics in roller bottles. Induction by NDV or Sendai virus was for 1 hour followed by a further 2 hours when the first IFN activity could be measured (data not shown).

Immunofluorescence: Differentiated or non-differentiated PC13 cells were treated with NDV (75 HAU/ml) for 1 hour. Immediately after infection, or 16-22 hours later, cells were treated with rabbit anti-NDV serum (a gift from Dr. D.P. Barlow) followed by fluorescein-labelled sheep anti-rabbit IgG (Wellcome Diagnostics).

Nuclei preparation: The method was that of Schriber and Weber (11). Nuclei were used fresh or stored at -70°C in RB (25% glycerol, 5mM Mg acetate, 50 mM Tris-HCl pH 8.0, 5 mM DTT, 0.1 mM EDTA).

DNase I digestion: Nuclei were taken up at 1 mg/ml DNA in RSB (1) and digested with 1 μ g/ml DNase I (BCL, EC 3.1.21.1) for increasing lengths of time. The reactions were stopped by the addition of SDS and EDTA to a final concentration of 0.5% SDS and 12.5 mM EDTA (12), and digested overnight with 100 μ g/ml proteinase K (BCL, EC 3.4.21.14), phenol-chloroform extracted and RNase A (BCL, EC 3.1.27.5) treated. 10 μ g/ml aliquots of the various DNA samples, digested with Eco RI (BRL) to completion, were loaded onto 1% agarose gels electrophoresed prior to blotting onto nitrocellulose paper (Schleicher and Schull) (13). Filters were hybridized to [³²P]-labelled nicktranslated probes (14). The probes used were mouse β interferon cDNA (pMIF 3/10, Pst I insert) (15) and human β interferon cDNA (D4P165, Msp I fragment) (16).

RESULTS AND DISCUSSION

DNase I Sensitivity of the Interferon β Gene In Undifferentiated PC13 Cell Nuclei.

Undifferentiated mouse teratocarcinoma cells fail to produce IFN upon viral induction. This could be because the virus fails to infect the cells and does not therefore induce the IFN system, but Figure 1 shows that undifferentiated PC13 cells, although not producing any IFN protein, do become infected by NDV and express virus proteins on the cellular outer membrane as judged by immunofluorescence. Fluorescence was seen in both undifferentiated (Figure 1a,b) and differentiated (Figure 1e,f) PC13 cells when expression of the virus proteins was allowed to occur. When virus was added only 1 hour prior to antibody treatment, very little immunofluorescence was detectable (Figure 1c,d,g,h). No immunofluorescence was observed if chick allantoic fluid and non-immune rabbit serum were substituted for virus and anti-NDV serum respectively (data not shown). Therefore, the fluorescence seen is due to newly synthesized virus proteins rather than the infecting virus particles. Thus, the failure to react to virus induction is not due to a failure in the infection by, or growth of the virus, but rather to some other process within the cell.

Nuclei were prepared as described in Materials and Methods both from non-induced and induced undifferentiated PC13 cells. These were digested with 1 μ g/ml DNase for up to 5 minutes. Under these conditions about 5% of the DNA is lost. The remaining DNA was extracted and digested with an excess of Eco RI to completion. The restricted DNA was run on an agarose gel, transferred to nitrocellulose paper and probed for mouse IFN β gene sequences. This revealed a single 4000 bp fragment in DNA from non-DNase I treated nuclei (Figure 2a, 0 minutes). As can be seen from Figure 2(a,b), this fragment is not degraded by DNase I in either non-induced or induced undifferentiated PC13 cells.

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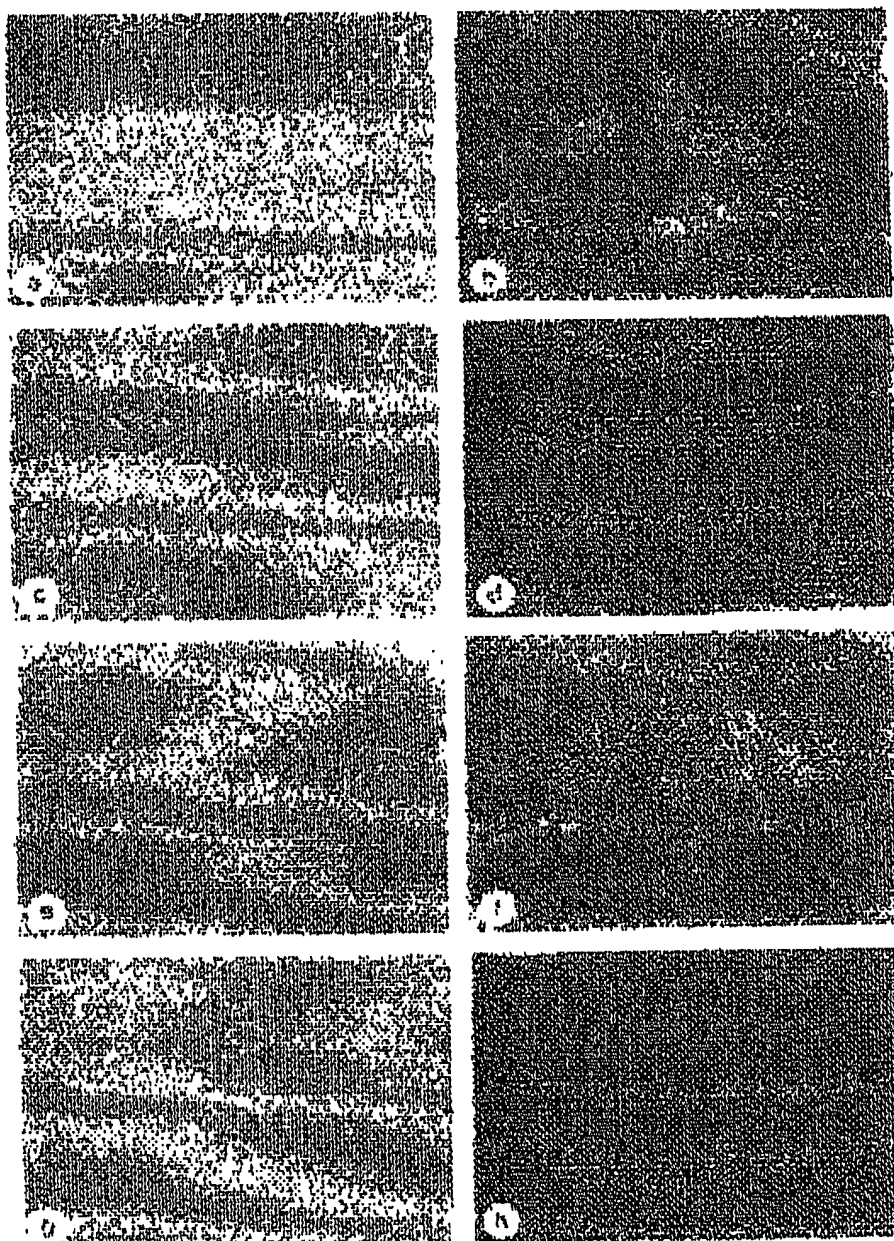


Figure 1: NDV-F Specific Immunofluorescence in PC13 Cells. Undifferentiated and differentiated PC13 cells were infected with NDV-F as described in the Materials and Methods. Cells were viewed under phase contrast (a,c,e,g) or UV illumination (b,d,f,h). 1a,b; undifferentiated cells, 16 hour incubation. 1c,d; undifferentiated cells, 1 hour incubation. 1e,f; differentiated cells, 20 hour incubation. 1g,h; differentiated cells, 1 hour incubation.

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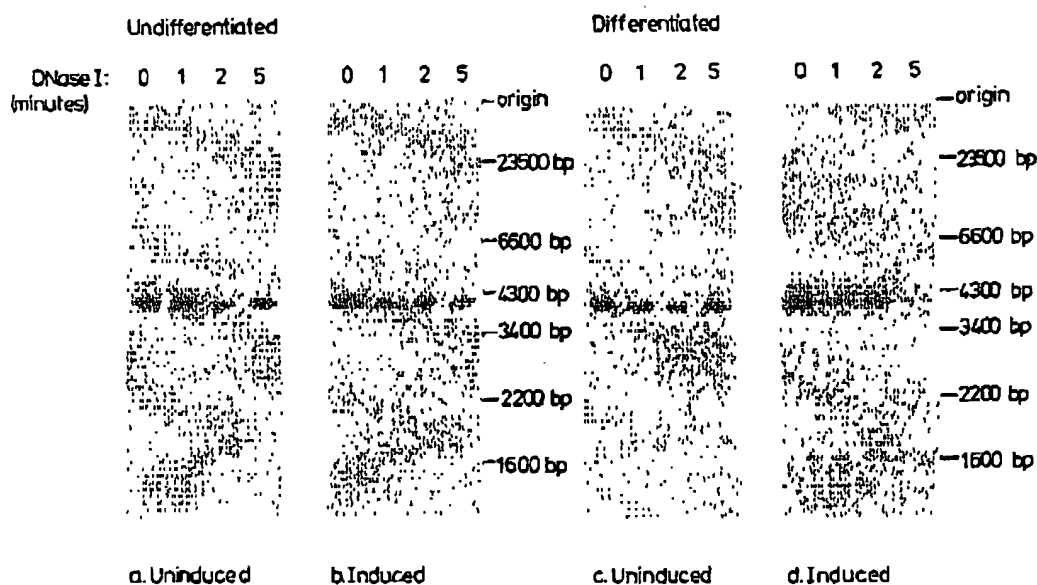


Figure 2: DNase I Sensitivity of the Interferon β Gene in PC13 Cells. Nuclei from the following cell types were digested with DNase I for 0, 1, 2 or 5 minutes prior to extraction, Eco RI digestion and analysis being carried out as described in Materials and Methods. 2a; non-induced, undifferentiated. 2b; induced, undifferentiated. 2c; non-induced, differentiated. 2d; induced, differentiated. In each case, the tracks are labelled with the time of DNase I digestion.

DNase I Sensitivity of the Interferon β Gene in Differentiated PC13 Cell Nuclei.

The experiment described above was repeated, but this time using non-induced and induced differentiated PC13 cells. These cells, unlike those prior to differentiation, are able to synthesize IFN after virus induction. When the DNA from DNase I-digested nuclei from non-induced cells was analyzed, no degradation of the 4000 bp fragment was seen (Figure 2c). However, upon induction of the cells, the IFN β gene does become DNase I sensitive as shown by the gradual loss of the 4000 bp fragment (Figure 2d).

Therefore, in the case of the mouse β IFN gene, the change in chromatin configuration that is detected by DNase I is a result of induction of an inducible cell. Neither the viral induction nor the differentiation alone causes the switch in chromatin structure to a more "open" and DNase I sensitive state. This is in contrast to what might be

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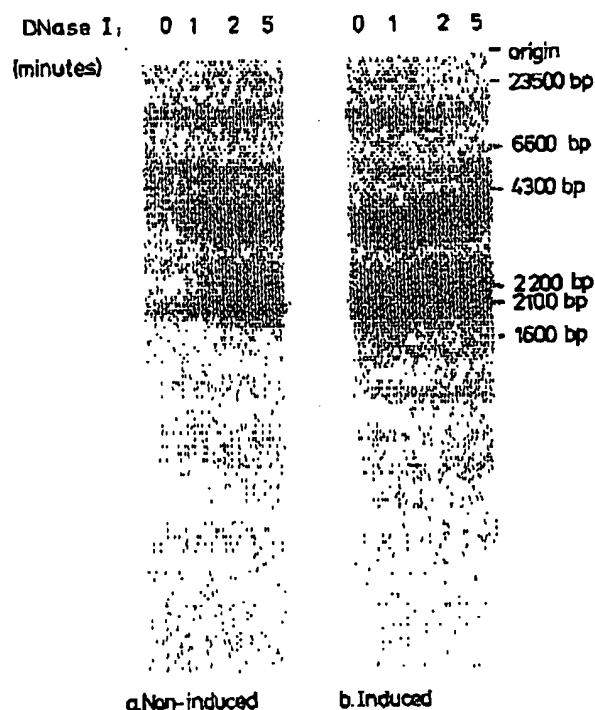


Figure 3: DNase I Sensitivity of the Interferon α Gene in MG63 Cells. Nuclei from the uninduced (3a) or induced (3b) MG63 cells were digested with DNase I for 0, 1, 2 or 5 minutes prior to extraction. Eco RI digestion and analysis being carried out as described previously. Tracks are labelled with the time of DNase I digestion.

expected if the change from DNase I resistance to sensitivity required DNA replication to occur.

DNase I Sensitivity of the Interferon α Gene in Human MG63 Cell Nuclei.

To investigate whether the change in DNase I sensitivity associated in PC13 cells only with induction of differentiated cells was confined to this cell line, a similar experiment was carried out using human MG63 cells. This differentiated cell line, derived from a human osteogenic carcinoma, synthesizes mostly IFN α upon virus induction. As shown in Figure 3, the single fragment of 1920 bp seen with the cDNA probe used, is not degraded in non-induced cells. In DNA isolated from DNase I treated induced MG63 cells, the IFN α gene is in a different chromatin conformation after induction, and this change is distinguished by DNase I sensitivity.

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Figure 5. Autoradiogram of 70S ribosomal proteins separated by 2D-electrophoresis after in vitro incubation with [γ^{32} P]-ATP, at 30°C, for 30 min in the presence of cyclic nucleotide independent casein kinase isolated from adrenal cortex. Exposure time: 10 hours.

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Interferon synthesis in the early post-implantation mouse embryo

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Abstract. A qualitative bioassay was adapted and used to determine the ability of the early post-implantation mouse embryo to synthesise interferon. Interferon production was not seen in any embryo tissue in the absence of an inducer and could only be detected in virus-induced tissue from the early 7th day of development. This induced interferon synthesis was initially confined to the trophoblast of the early 7th day embryo. It was then found in tissues of both trophoblast and inner cell mass origin in the early 8th day, and subsequently, in derivatives of the embryonic ectoderm in the 13th-day embryo.

Introduction

Interferons are a heterogeneous family of secreted cell proteins defined by their antiviral activity, but they are also capable of exerting other, apparently unrelated, effects on the cell [4, 5]. The role of interferon in mediating the cellular inhibition of virus growth is now quite well understood [3, 30]. Other established effects of interferon that are less well understood include the inhibition of cell growth [35] and the regulation of the immune system [9]. In addition, more recent work on the Friend leukaemic as well as other cell systems [7, 10, 19, 20, 32] and levels of the interferon-associated enzyme, 2–5 A synthetase, in normal and differentiating cells [34] has suggested that interferons may also be involved in regulating some aspects of normal cell differentiation. Further support for this hypothesis has come from the demonstration that the interferon system is developmentally regulated in teratocarcinoma cells, since both interferon production and sensitivity are only seen in the differentiated cell [6]. Since undifferentiated teratocarcinoma cells are analogous to the pluripotent cells of the early mouse embryo [16, 23], the results from the teratocarcinoma cell system suggest that the interferon system may be similarly developmentally regulated in the early mouse embryo, appearing as cell differentiation occurs.

As a preliminary to understanding the role, if any, of interferon during normal differentiation in early mouse embryogenesis, we tested the competence of early embryonic and associated maternal tissues to synthesise interferon. Hitherto, the small amount of tissue available has precluded

direct examination of interferon production and action in early embryos by conventional interferon assays. However, results from indirect studies using *in vitro* cultures established from embryos of different ages have suggested that interferon production and action are reduced in early embryos [11, 18, 24]. We used a sensitive, qualitative bioassay to detect directly interferon production from embryonic tissue fragments approximately 100–500 μ m in diameter. The results show that interferon synthesis in the absence of an inducer (i.e. constitutive synthesis) is absent in embryonic and associated maternal tissue in the early and late post-implantation embryo. Virus-induced interferon production could not be elicited in any embryonic tissue until the 7th day of development. This induced synthesis developed to maximum levels (compared to control virus-induced maternal tissue) in the majority of embryonic tissues during the 7th–8th day, but remained absent in one extra-embryonic tissue (visceral extra-embryonic endoderm) and the embryonic ectoderm (which, at this stage, is the only source of the embryo proper). By the 13th day, however, maximum levels of induced interferon synthesis could be elicited from both embryonic and extra-embryonic tissues.

Methods

Mouse embryos. Embryos at different stages of development were obtained from natural matings between strain 129J/Sv females and F₁(C57BL/CBA) males. The first day of development is the day of the formation of the vaginal plug. Pregnant mice between the 7th and 13th day of development were killed by cervical fracture, and their embryos were removed into alpha medium (as has been described in [33], but lacking nucleotides and deoxynucleotides and containing 5% (v/v) heat-inactivated foetal bovine serum, pre-equilibrated with 5% CO₂ in air). Extra-embryonic and embryonic tissues were dissected into fragments approximately 100–500 μ m in diameter using hand-pulled glass needles. Sizes were checked using a standard graticule and high-power microscope, and individual fragments were placed into separate wells of a Terasaki microtitre plate. Eight pregnant mice with a litter size of between five and nine embryos were used for each time point, and no more than five tissue fragments of any type were obtained from any one embryo. Similar-sized tissue fragments from the maternal decidua and uterus were also obtained during each dissection. Using identical dissection methods, it has been demonstrated that the distribution of reactivity to a mono-

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clonal antibody on dissected embryo pieces is identical to that in whole sectioned embryos on the 7th day of development [29]. Therefore, it is likely that these dissections adequately separate the cell layers of the early embryo. Dissections were carried out between mid-morning and mid-afternoon, depending on the stage required, and the tissue fragments were incorporated into the interferon assay approximately 4–5 h later.

L929 Cells. Cells were grown routinely in 1-l glass bottles in the Glasgow modification of Eagle's medium (GMEM) plus 10% (v/v) new-born calf serum and passaged every 5 days. For use in the interferon assay, 1×10^6 cells were seeded into a 5-cm tissue-culture plastic dish which had been previously coated with gelatin (a 1% solution of gelatin was applied for 1 h at 4° C and then removed, and the dish air dried) and incubated overnight. The use of gelatinised dishes preserved the monolayer intact throughout the assay and promoted a more uniform dispersion of the cells over the culture dish. The latter aided visualisation of the assay endpoint.

Agar medium. The agar medium used consisted of 0.3% agar, 5% (v/v) heat-inactivated foetal bovine serum (fbs), 50 mM NaHCO₃, 0.5 units/ml benzylpenicillin, 0.5 µg/ml streptomycin, $1 \times$ alpha medium and 20 mM glutamine. Agar medium was prepared from sterile stock solutions as required. All solutions except the agar were mixed and kept at 37° C. The agar was then liquified by boiling and cooled to 45° C before addition to the remaining solutions. The mixture was allowed to cool to 37° C before use in the assay.

Virus stocks: Newcastle disease virus-strain F. Newcastle Disease Virus (NDV) is an avian paramyxovirus known to induce high yields of interferon in mouse cells. Strain F (NDV-F) is avirulent in mouse cells and will not lyse them [22]. Stock virus was obtained by injecting, 1,000 haemagglutinin units (HAU) of NDV-F into the allantoic cavity of 10-day embryonated hen eggs. After incubation for 60 h at 37° C, the allantoic fluid was harvested, cleared by centrifugation at 10,000 rpm for 30 min, dispensed in 50-µl aliquots, snap frozen and stored at -70° C. The stock (10,000 HAU units/ml) was used without further purification at a dilution of 1:1,000 to induce interferon synthesis in embryonic tissue fragments. This concentration induces maximum interferon synthesis in L929 cells (data not shown).

Encephalomyocarditis virus. Encephalomyocarditis virus (EMC) is a picornavirus which can replicate in and lyse mouse cells, but whose cytolytic action is inhibited by the pretreatment of the cell with interferon. Stock virus was prepared from a 1-l flask of confluent L929 cells. Growth medium was removed, and the monolayer was washed once with phosphate buffered saline (136 mM NaCl, 2.68 mM KCl, 8.1 mM Na₂HPO₄, 1.46 mM KH₂PO₄; PBS) before the addition of 2 ml serum-free growth medium containing 1×10^9 plaque-forming units (pfu) of EMC. After 1 h, the cells were refed with 20 ml growth medium and left for 20 h, at which point cell lysis was evident. The supernatant was harvested, cleared by centrifugation at 10,000 rpm for 30 min, dispensed into 50 µl aliquots, snap frozen and stored at -70° C. Virus concentration was determined by

plaque formation on L929 cells as 4×10^8 pfu/ml, and the virus was used in the assay at a 1:100 dilution without further purification. Under the conditions used in the interferon assay described, 100% of the L929 indicator monolayer cells were lysed when infected with 4 pfu per cell for 16 h.

NDV-F antiserum. NDV-F antiserum was obtained following the injection of partially purified virus protein [2] into rabbits. Serum was collected 28 days later, heat inactivated before use and titred by two methods:

1. Standard haemagglutinin-inhibition assay when the titre was found to be 1:640.

2. Neutralisation of interferon-inducing ability of NDV-F virus. NDV-F (0.1 HAU) was mixed with different dilutions of antiserum for 1 h at room temperature, and the ability of the virus to induce interferon in L929 cells was then assayed in a standard assay [1]. The complete neutralisation of interferon-inducing ability was obtained with a 1:100 dilution, and the antiserum was diluted 1:10 for use in the assay.

Interferon assay. The interferon assay, modified from one which has been described previously [27], was designed to detect qualitatively interferon production either from small tissue fragments or from single cells. It involves three steps:

1. The infection of tissue fragments with an interferon-inducing virus (i.e. NDV-F).

2. The incubation of these induced fragments adjacent to an indicator cell monolayer. During this period, interferon produced by the fragments is absorbed by the indicator cells.

3. The challenge of the indicator cell monolayer with a cytolytic virus (i.e. EMC) to reveal foci of cells exposed to interferon during step 2.

All incubations were carried out in a gassed (5% CO₂ in air) and humidified incubator at 37° C. Step 1 was performed with the tissue fragments placed separately in individual wells of a Terasaki microtitre plate in alpha medium containing 5% heat-inactivated fbs. The medium was changed using a finely drawn glass capillary controlled by mouth pipette, with the sample viewed by a dissecting microscope.

Test fragments were incubated for 1 h in 10 µl medium plus 0.1 HAU NDV-F, washed three times in medium alone and then incubated for a further hour in medium plus a 1:10 dilution of NDV-F antiserum. These induced tissue fragments were then placed inside circumscribed areas on top of an L929 cell monolayer and fixed in position with a drop of agar medium. The monolayer was then covered with 1.5 ml agar medium and incubated for 20 h. The purpose of the agar medium was to fix the samples in a defined position on the monolayer and limit the diffusion of interferon to the immediate area of the test fragment. The agar medium and test fragments were then dislodged from the indicator cell monolayer by tapping the assay dish at an angle of 45° and were removed by aspiration. The monolayer was washed twice with prewarmed (37° C) PBS and then challenged with EMC virus: 4×10^6 pfu EMC in 100 µl serum-free medium was added to each monolayer for 1 h. The dish was then refed with 4 ml growth medium and incubated for 16 h. To reveal foci of interferon-protected cells, the indicator monolayer was washed once with PBS, fixed in 100% methanol for 1 min and then stained with 0.1% crystal violet for 10 min.

Fig. 1. negative at the tissue pretreatment into the interferon of the virus

Tissue from day 10 Circled while NDV-F in culture

Serial quantitative synthesis of interferon in the

Serial and maintenance of the virus in the

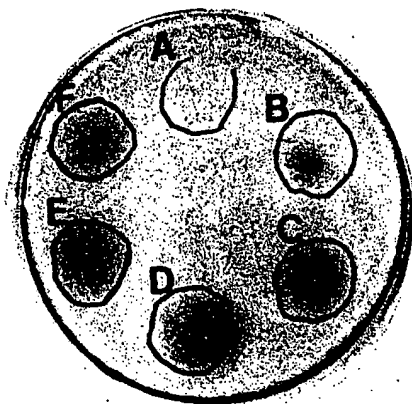


Fig. 1. A typical assay plate showing positive (circles B-F) and negative (circle A) interferon synthesis by the parietal endoderm at the 13th day of development. See Methods for the assay details. Tissue fragments were either incorporated into the assay without pretreatment (circle A) or induced with virus and then incorporated into the assay (circles B-F). Only the virus-induced fragments synthesised interferon and produced foci of protected cells. The diameter of the foci within circles B-F (5-7 mm) did not correlate with the variation in size (100-500 μ m) of the induced tissue fragments

The appearance of the indicator monolayer at completion of the assay is shown in Fig. 1. Six tissue fragments from the extra-embryonic parietal endoderm at the 13th day of development were placed in the centre of each circle. Circle A contained a fragment not exposed to NDV-F, while circles B-F each contained a fragment exposed to NDV-F. The foci of interferon-protected cells 5-7 mm in diameter (containing 2,500-3,500 cells) can only be seen in circles B-F.

Sensitivity of the interferon assay. Interferon is routinely quantified by assaying the inhibition of viral nucleic acid synthesis in cells pretreated with interferon [1]. When L929 cells are used in such an assay, they can detect 1.0 International Reference Research Unit (IRRU) of mouse interferon. (Interferon standards were obtained from the National Institute for Biological Standards, London, UK.) The qualitative assay described here can detect 0.5 IRRU mouse interferon when applied directly to the agar medium above the indicator cells (data not shown).

Source of materials. The mouse strains, 129J Sv females and F₁ (C57BL/CBA) males, were obtained from the animal house, Oxford University, and "half-lop" rabbits were obtained from Hylyne, Surrey, UK. Tissue culture medium, new born calf serum and fbs were obtained from Flow Laboratories, Irvine, UK. Terasaki microtitre plates and 5-cm plastic dishes were obtained from Falcon Plastics, Scientific Supplies, London, UK. L929 cells and Freund's adjuvant were obtained from Gibco Biocult, Paisley, Scotland. Gelatin (swine skin type III) was obtained from Sigma Chemicals, Dorset, UK. Agar was obtained from Difco Laboratories, Surrey, UK, and benzylpenicillin and streptomycin were obtained from Glaxo Laboratories, Middlesex, UK. Crystal Violet was obtained from BDH Chemicals, Dorset, UK.

Results

Using a qualitative bioassay, we assayed maternal and embryonic tissues for both constitutive interferon synthesis and competency to be induced to synthesise interferon. Test tissues were obtained at the 7th-8th to and 13th days of development from the maternal uterus and decidua of strain 129J/Sv females and embryos resulting from matings between 129J/Sv females and F₁ (C57BL/CBA) males. Each tissue was dissected into fragments 100-500 μ m in diameter. Samples from the same tissue were either incorporated into the bioassay without pretreatment in order to measure constitutive interferon synthesis or exposed to an inducing virus and then incorporated into the bioassay in order to measure competency for interferon induction.

Seventh to eighth day of development. The results obtained from the early post-implantation mouse embryo are shown in Table 1 and Fig. 2. Table 1 shows both constitutive and induced interferon synthesis in separate tissues of the embryo and the maternal tissues enclosing the developing embryo, at the early 7th, late 7th and early 8th day of development. Figure 2 shows the development of virus-induced interferon synthesis in these stages of embryogenesis.

The data in Table 1 show that constitutive interferon synthesis (i.e. synthesis of interferon in the absence of an inducer) was not seen in any embryonic or maternal tissue at this time. Measurements of both constitutive and induced interferon synthesis were performed upon fragments obtained from the same tissue, using assays carried out at the same time as those in which induced fragments gave positive results (see Table 1, induced columns).

In contrast to the results obtained for constitutive synthesis, it was possible to induce the early post-implantation embryo and associated maternal tissue to synthesise interferon upon virus infection. The extent of this induced synthesis is described in Fig. 2 as nil (none of the induced tissue fragments included in the assay produced a positive result), low (20%-30% of induced fragments produced positive results) or high (60%-100% of induced fragments produced positive results). The measurement of induced interferon synthesis in these embryonic stages highlights four main points:

1. Induced synthesis is initially absent in most tissues of the early 7th-day embryo, with the exception of the trophoblast, which shows a low level (33%) of inducible synthesis. It should also be noted that control virus-induced maternal tissues early in the 7th day showed a reduced level of induced synthesis compared to the late 7th and early 8th day of development (approximately 35%, compared to greater than 70% at later stages). These results were seen in two separate assays of early 7th-day material obtained from different matings.

2. Induced synthesis is developmentally regulated, reaching high levels in late 7th-day and early 8th-day embryos in tissues which initially showed nil or low levels of induction (Fig. 2). By the early 8th day, all tissues, with the exception of the visceral extra-embryonic endoderm (V. ex. END) and the embryonic ectoderm (emb. ECT), could be induced to produce interferon.

3. Non-inducible tissues do not inhibit interferon synthesis from inducible tissues. Two tissues remained non-inducible at the early 8th day of development (V. ex. END, emb. ECT). When these tissues were assayed together with

Table 1. Constitutive and induced interferon synthesis in the tissues of the early post-implantation mouse embryo at the early 7th, late 7th and early 8th day of development

	Early 7th day				Late 7th day				Early 7th day			
	Constitutive		Induced		Constitutive		Induced		Constitutive		Induced	
	%	(n)	%	(n)	%	(n)	%	(n)	%	(n)	%	(n)
Embryo												
EPC	0	(4)	0	(24)	0	(4)	75	(12)	0	(4)	71	(7)
TB	0	(5)	33	(22)	0 ^a	(4)	75	(8)	0	(4)	80	(15)
PE	0	(4)	0	(10)					0	(5)	0	(10)
V. ex. END	0	(4)	0	(19)	0	(4)	11	(18)	0	(5)	33	(6)
ex. ECT	0	(4)	0	(20)					0 ^b	(5)	73 ^b	(11)
V. END	0	(4)	0	(21)	0	(5)	7	(15)	0	(5)	0	(12)
emb. ECT	0	(4)	0	(13)	0	(4)	0	(10)				
Maternal												
Myometrium	0	(10)	36	(27)	0	(10)	92	(12)	0	(10)	73	(21)
Decidua	0	(10)	40	(22)	0	(10)	100	(10)	0	(10)	70	(27)

The percentage of tissue fragments that produced a positive result in the interferon assay is given in each column. Each result is the average of two assays conducted on a number of fragments (*n*) from two separate matings. Tissue fragments (100–500 µm in diameter) were either incorporated into the interferon assay without pretreatment to measure constitutive synthesis or induced with virus and then incorporated into the assay to measure induced synthesis.

EPC, ectoplacental cone; TB, trophoblast of the parietal yolk sac; PE, parietal endoderm; V. ex. END, visceral extra-embryonic endoderm; ex. ECT, extra-embryonic ectoderm; V. END, visceral embryonic endoderm; emb. ECT, embryonic ectoderm.

^a Results which are bracketed together were obtained from tissues assayed jointly.

^b With the manual dissection employed here, the mesoderm arising at the early primitive streak stage is included with the V. END.

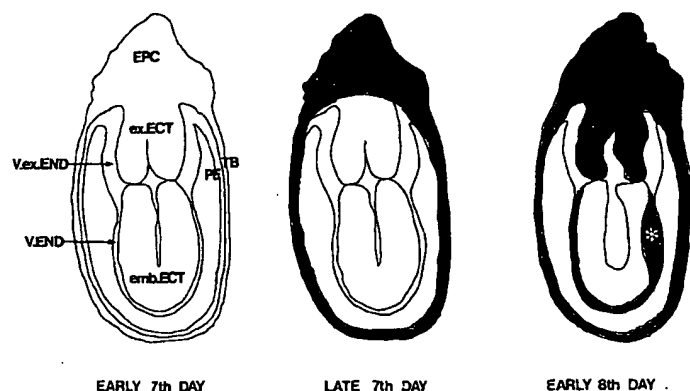


Fig. 2. Virus-induced interferon synthesis in the mouse embryo at the early 7th, late 7th and early 8th day of development based on data presented in Table 1. *Unshaded areas* represent tissues which were non-inducible, *light shading* represents tissues with a low level of inducibility (10%–30% of tissue fragments produced positive results in the assay), and *heavy shading* represents tissues with a high level of inducibility (60%–100% of tissue fragments produced positive results in the assay). See Methods for the interferon-assay details. For abbreviations, see legend of Table 1. With the manual dissection employed here, the mesoderm arising at the early primitive streak stage is included with the V. END.*

their neighbouring tissues (i.e. V. ex. END plus extra embryonic ectoderm and emb. ECT plus visceral endoderm) the interferon response was unaffected (91% and 82%, respectively).

4. Early differentiated tissues are non-inducible. In the early 7th-day embryo, induced synthesis could not be elicited from any differentiated tissue except the trophoblast of the parietal yolk sac (PYS). By early in the 8th day, most of the differentiated tissues could be induced, although the V. ex. END remained an exception. The undifferentiated pluripotent embryonic ectoderm, which is the source of the embryo proper, could not be induced to synthesise interferon, even when taken from early 8th-day embryo.

Thirteenth day of development. The results obtained from the late post-implantation embryo and associated maternal tissues are shown in Table 2 and Fig. 3. Table 2 shows both constitutive and induced interferon synthesis, and Figure 2 shows the arrangement of the embryo and extra-embryonic membranes, and the level of induced interferon synthesis in these tissues. These data were obtained as previously described for the early post-implantation embryo.

The data in Table 2 show that, as with the early post-implantation embryo, constitutive interferon synthesis was not seen in any embryonic or maternal tissue. Table 2 and Fig. 3 show that the 13th-day embryo, the three surrounding extra-embryonic membranes and the associated mater-

Table 2. Constitutive and induced interferon synthesis in the tissues of the late post-implantation mouse embryo

	13th day of development			
	Constitutive		Induced	
	%	(n)	%	(n)
Embryo				
Placenta	0	(6)	100	(11)
Trophoblast	0	(6)	4	(31)
Parietal	0	(6)	76	(31)
Endoderm				
Visceral	0	(6)	70	(30)
Yolk sac				
Amnion	0	(6)	67	(30)
Neural tissue	0	(6)	67	(30)
Heart tissue	0	(6)	50	(19)
Gut tissue	0	(6)	82	(17)
Maternal				
Myometrium	0	(10)	75	(26)

Each result is the average of three separate assays conducted on a number of fragments (n) from three separate matings. The legend is as for Table 1



13th DAY OF DEVELOPMENT

Fig. 3. Virus-induced interferon synthesis in the mouse embryo and extra-embryonic membranes at the 13th day of development based on data presented in Table 2. Abbreviations as for Table 1 plus in addition, PLAC, placenta; VYS, visceral yolk sac; AM, amnion; NT, neural tissue; HT, heart tissue; GT, gut tissue

nal tissue could all be induced to synthesise high levels of interferon upon virus infection (60%–100% of the fragments were positive).

The three extra-embryonic membranes are all bilayered, and the outermost membrane, the PYS, is composed of an inner layer of parietal endoderm cells and an outer layer of trophoblast cells. The PYS atrophies during the last third of embryonic development. In the embryos used in this study, the trophoblast was present at the 13th day, but was clearly reduced by the 15th day of development. The

trophoblast is, therefore, the outermost embryonic cell layer of the 13th-day and early post-implantation embryo, and is in close contact with the maternal decidua and blood. The nucleated cells of maternal blood are inducible by virus and could potentially produce false positives within the assay. However, the negative results obtained with 13th-day PYS trophoblast tissue (0%, 11% and 0% in three separate assays) suggest that the contamination of this embryonic tissue with maternal blood results in negligible interference with the assay results.

Discussion

Although much is known concerning interferon induction, this is the first report describing interferon synthesis in a developing embryo. We show here that, in embryos resulting from matings between 129J/Sv females and F₁ (C57BL/CBA) males, the competence to synthesise interferon in response to a virus inducer is developmentally regulated. Furthermore, interferon synthesis in the absence of an inducer (i.e. constitutive synthesis) is not seen in any embryonic or maternal tissue before the 13th day of development.

The majority of embryonic tissues present early in the 7th day of development are either non-inducible by virus (e.g. ectoplacental cone, parietal endoderm, visceral endoderm, visceral extra-embryonic endoderm, embryonic ectoderm and extra-embryonic ectoderm) or only able to synthesise low levels of interferon (e.g. the trophoblast). This situation alters by early in the 8th day, when only two tissues (the visceral extra-embryonic endoderm and the embryonic ectoderm) remain non-inducible. Further change occurs by the 13th day of development when high levels of interferon synthesis can be induced in all embryonic tissues tested, with the exception of the trophoblast (Tables 1, 2). The lack of inducibility of the 13th-day trophoblast contrasts with the high level seen in the 8th-day trophoblast and is probably related to the atrophy and removal of this tissue which occurs during the 15th–16th day of development. The competence of early embryonic tissue to synthesise interferon in response to virus induction thus arises non-synchronously in most differentiated tissue, while remaining absent from undifferentiated (i.e. pluripotent) tissue. This behaviour resembles that described [6] in differentiating teratocarcinoma cells. Undifferentiated teratocarcinoma cells could not be induced to produce interferon until they were allowed to differentiate in vitro, when these cells could be induced.

Two cell types, distinguished by morphological and biochemical criteria, are initially established in the pre-implantation mouse embryo and are termed the inner cell mass (ICM) and the trophectoderm (TE). Tissues present in the early post-implantation embryo are derived from either of these cell types [15]. The appearance of interferon inducibility in the post-implantation embryo is not restricted to cells of one lineage. Instead, as seen in Table 1, tissues of both TE origin (e.g. ectoplacental cone) and ICM origin (e.g. visceral endoderm) show equally high levels of inducibility by the early 8th day of development. In addition, all tissues derived from any one lineage are not equally inducible. For example, the visceral extra-embryonic endoderm, the visceral endoderm and the parietal endoderm all originate from the primitive endoderm of the late 4th-day embryo [17]. Of these, the former is non-inducible, while the two latter tissues are highly inducible at the 8th day

of development (Table 1). Since all three tissues originate at the same developmental age and from the same precursor population, it is unlikely that a trivial explanation, such as differential viability, can explain the lack of interferon induction in the visceral extra-embryonic endoderm. The restriction of interferon inducibility in the 8th-day visceral endoderm to those cells which overlie the embryonic ectoderm is identical to the restriction of alphafoetoprotein synthesis in visceral endoderm which is observed at this time [13]. It is therefore possible that the tissue interactions which are thought to modulate the synthesis of alphafoetoprotein [12] also influence interferon inducibility.

The synthesis of interferon in the absence of an inducer (i.e. constitutive synthesis) was not seen in embryonic or maternal tissue at the 7th–8th or the 13th day of development (Tables 1, 2). These results from the 13th day of development contrast with those which have been obtained from NIH Swiss mouse embryos [14]. In this strain, high levels of constitutively produced interferon have been detected in the placenta from the 10th day until term. Differences between lines of mice in their response to viral induction of interferon synthesis have been recorded for adult mice [8]. It is therefore possible that the differences between constitutive interferon synthesis in extra-embryonic tissues of NIH Swiss embryos and the embryos described here are strain dependent.

Two controls were introduced in order to monitor the efficiency of the bioassay. The first made use of similar-sized tissue fragments taken from the uterus myometrium and deciduum. The second involved the measurement of constitutive and induced interferon synthesis on different fragments taken from the same tissue. Thus, negative results were always obtained under conditions when control-induced tissue produced positive results in assays conducted at the same time. Therefore, we feel it is unlikely that the negative results described here have a trivial explanation, such as lack of viability in vitro. The control virus-induced maternal tissues (i.e. the uterus myometrium and decidua) showed a reduced level of induced interferon synthesis (i.e. 36%–40%) compared to results obtained from later gestation stages (i.e. 73%–100%). It is not clear why this is so, or if it indicates a depression of induced interferon synthesis in the embryo as well. However, this result does not reflect variability in the assay itself, since this result could only be seen in maternal tissues from the early 7th day of development and not in later development stages.

The data show that some tissues of the early embryo can be induced to synthesise interferon, whilst neighbouring tissues are non-inducible. It is not known whether this lesion in the non-inducible cells is at the level of virus entry and inducer formation or at the level of induction and expression of the interferon gene itself. However, it is known that lytic virus replication, and therefore probably inducer formation, does occur in undifferentiated teratocarcinoma cells [6] which are considered to be analogous to early undifferentiated embryo cells. In addition, the results in Table 1 show that interferon synthesis is not induced in isolated V. ex. END or in emb. ECT, but can be induced in aggregates of these tissues with their neighbouring tissues. This suggests that the inability to produce interferon is not due to a diffusible antagonist of either the inducer or interferon itself.

The ability of tissues of the early post-implantation embryo to synthesise interferon in response to virus induction

suggests that this mechanism is of importance in eliminating pathogenic virus from the foetus. The early embryo itself has no immune defence mechanisms: immunocompetent cells do not develop until the 14th–15th day of development [21, 31] and maternal interferon is not thought to cross the placental barrier [28]. We have shown that the post-implantation embryo from the late 7th day of development can synthesise interferon in response to a virus infection. However, interferons are known to exert pleiotropic effects on the cell, including the inhibition of cell proliferation [35], and cells of the post-implantation embryo are known to be sensitive to these anti-proliferative effects [11]. It is therefore possible that the teratogenic effects produced by some virus infections of the foetus [25, 26] may be attributed both to viral cytolysis and the anti-proliferative actions of interferon.

The role that interferon and interferon-associated enzymes may play in regulating cell differentiation is beginning to be studied. In this study, we show that upon appropriate stimulation, the embryo can synthesise interferon from the late 7th day of development. This raises the possibility that embryonic interferon may influence some events in embryogenesis.

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into an exon or intron of an actively transcribed endogenous gene, thereby simultaneously disrupting the gene and acting as a locus-specific marker of the gene. This would greatly facilitate the identification and cloning of the disrupted genes, a task that is not necessarily straightforward following chemical mutagenesis. In practice, hundreds of transgenic embryos, each carrying multiple integration sites, could be generated per day and screened for GFP expression. A huge advantage of using this approach in frog embryos is that they develop externally, therefore GFP expression can be assayed in living embryos at any stage. Most embryos will not express the marker gene. These will be discarded and only the few that express will be nurtured to maturity, thus greatly reducing the number of embryos that must be carried to the next generation. Preliminary experiments in *X. laevis* strongly suggest that using a gene trap approach will be productive (O. Bronchain and E. Amaya, unpublished).

The powerful manipulations that one can perform on amphibian embryos have been used to reveal important principles about develop-

ment for over a century. As we approach the next century, it appears that it will now be possible to overlay this rich embryological history with the power of genetic manipulations, creating an armamentarium of approaches as we look towards revealing a new generation of concepts about vertebrate embryonic development.

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Double-stranded RNA as a mediator in sequence-specific genetic silencing and co-suppression

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Many fundamental natural processes have been uncovered not by pre-planned scientific enquiry, but serendipitously by engineers and scientists who observed unexpected consequences of their manipulations. Biologists routinely use engineering to manipulate the expression of specific genes and, thus, understand (or benefit from) their function. Sometimes we wish to make a particular gene silent; at other times we want the genes to 'talk' more loudly. Attempts at silencing have often employed an antisense strategy of introducing single-stranded nucleic acid from the noncoding strand to sequester or modify the

native transcript, thereby preventing accumulation of the corresponding protein. Conversely, by introducing extra copies of a specific gene, one might expect in many cases to over-produce the corresponding mRNA and protein products. Although these techniques have been successful in numerous applications, a body of literature is emerging that documents certain cases in which unexpected outcomes of these manipulations are seen in organisms as diverse as nematodes and plants. These observations encompass 'transgene silencing' (a failure to express certain multi-copy transgenes) and co-suppression

(the ability of a 'sense' transgene to interfere with the activity of the endogenous genetic locus). Certain of these phenomena are thought to involve direct DNA–DNA interactions, whereas others have been proposed to require an RNA effector molecule. The structure and mechanistic properties of RNAs mediating the latter type of co-suppression have yet to be elucidated. Here, we discuss the possibility that double-stranded RNA (dsRNA), rather than sense or antisense single-stranded RNAs alone, is the effector molecule responsible for RNA-mediated silencing and co-suppression.

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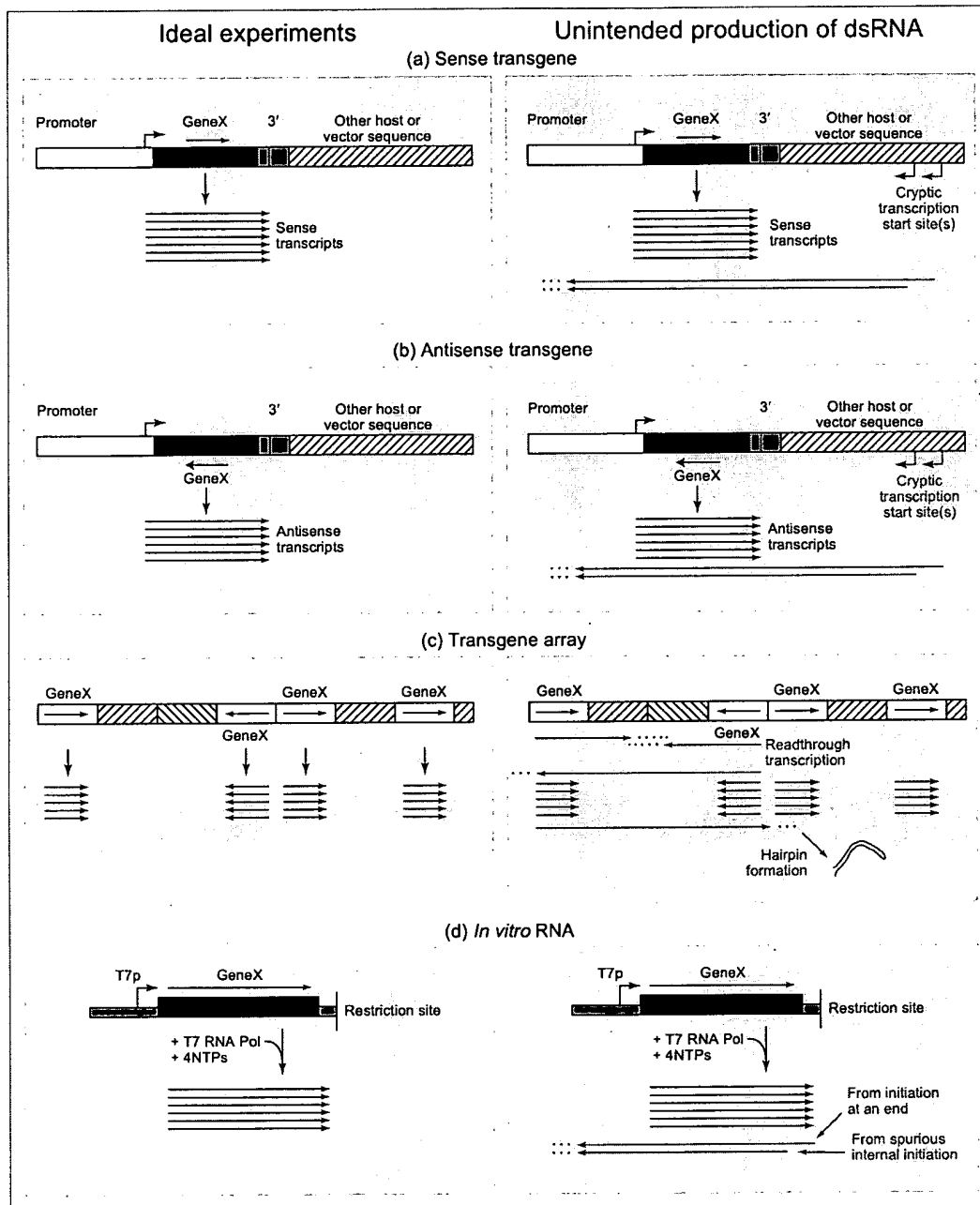


FIGURE 1. Unintended production of double-stranded (ds) RNA. Left, a series of theoretical experiments that might be designed to produce a pure population of single-stranded RNA. Right, how a low level of dsRNA could also be produced in each case. (a) A transgene designed to produce 'sense' RNA is transcribed at low level from a cryptic (or natural) start site on the opposite strand. Hybridization to 'sense' transcripts from the same template would result in dsRNA. (b) A transgene designed to produce 'antisense' could similarly be subject to low-level transcription on the opposite strand, with dsRNA resulting from hybridization of the newly transcribed RNAs. (Note that antisense RNA might alternatively hybridize with the endogenous chromosomal transcript to make dsRNA; it is not clear, however, that sense and antisense RNAs synthesized at distant nuclear sites would form dsRNA and be capable of interference.) (c) A transgene array containing tandem and inverted copies of a DNA construct ('geneX') might be expected to produce only one strand of RNA. Note, however, that readthrough of the geneX terminator would produce RNA with an inverted repeat structure. This RNA could undergo intramolecular hybridization to produce a predominantly double-stranded hairpin. (d) During *in vitro* synthesis of RNA, transcription initiates primarily at the bacteriophage RNA-polymerase promoter. Initiation can, however, also occur at internal sites and template ends, which leads to some inclusion of dsRNA in 'sense' and 'antisense' RNA preparations.

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RNA-mediated genetic interference (RNAi) in the nematode *C. elegans*

Several years ago it was reported that antisense RNA targeted to specific endogenous genes in *Caenorhabditis elegans*, when either expressed from a transgene¹ or injected directly into the worm's gonad², could phenocopy a null or hypomorphic mutation in the targeted gene. Surprisingly, both reports indicated that sense transcripts also were effective in producing the targeted phenotype. These observations were difficult to explain using a conventional model of antisense sequences inactivating the sense transcript. Recently, at least part of this mystery was solved by the discovery that much, if not all, of the genetic interference from injected 'sense' and 'antisense' RNA is actually mediated by double-stranded RNA (dsRNA) that is present at a low level in all *in vitro* RNA syntheses because of the non-specific activity of RNA polymerases³ (Fig. 1). Highly purified preparations of antisense (and sense) RNAs had negligible effects, whereas dsRNA exhibited potent and specific interference against any of a variety of targeted genes. The potency of RNAi in worms points to the existence of novel mechanism(s)³. Even with an abundantly transcribed target (several thousand mRNA copies per cell), a few molecules of dsRNA per cell can produce specific inhibition. This would not be expected from a simple antisense mechanism; there is simply not enough material to bind to all the endogenous mRNA targeted for destruction. These results generated two fundamental questions: how can dsRNA mediate gene-specific interference; and what is the physiological purpose of this process?

RNA-mediated silencing and co-suppression in plants

A second body of work on RNA-mediated interference comes from the plant world⁴. In the late 1980s, plant researchers were surprised to find that the introduction of certain transgenes into plants can result in homology-dependent silencing of an endogenous locus (rather than overexpression of the coding region of interest). This phenomenon is referred to as co-suppression. Not all transgenes cause this effect; there is no current basis for predicting which would and which would not. Gene silencing in plants has been proposed

to encompass a variety of different mechanisms^{4,5}, including some that act by direct DNA-DNA interaction and others that involve interference by an RNA product of the transgene. Strong evidence for the latter class of mechanisms comes from experiments in which RNAs are introduced in the absence of a DNA template (using RNA viruses as vectors); the ability of viral RNAs to interfere with a homologous gene in the plant genome is one of the strongest arguments for the existence of RNA-mediated silencing mechanisms⁶⁻⁸.

The literature contains a few clues as to the nature of the interfering RNA. In certain cases, co-suppression is correlated with high-level transcription of the transgene^{9,10}. Given recent results in *C. elegans*, we pose the possibility that transcription along the antisense strand of a transgene could result in low levels of interfering dsRNA (Fig. 1). Such transcription might be low-level synthesis directed by sequences within the vector or flanking regions at the site of integration (e.g. see Ref. 11). Significantly, Que *et al.*¹⁰ reported that, whereas co-suppression was associated with accumulation of transcripts at high concentrations from single-copy transgenes, inversely repeated transgenes could cause co-suppression, irrespective of promoter strength or level of the transgene mRNA. Transcripts from inversely repeated transgenes would be expected to produce a double-stranded structure. Experiments with chimeric RNA viruses^{7,8} might similarly point toward a dsRNA involvement; in these experiments, the viral RNA replicase copies the chimeric RNA in the cytoplasm, generating both sense and antisense material.

Similarities between nematodes and plants

RNAi in worms and co-suppression in plants share some striking similarities. Both are cases of gene-specific interference. dsRNA has been shown to be the agent of interference in nematodes and, as suggested above, there is some indication that dsRNA could also be responsible for co-suppression in plants. Perhaps the most interesting common characteristic is that the phenomenon can spread from the site of interfering RNA synthesis or application. In worms, the dsRNA mix can be injected into the body cavity, where it can produce an

interfering effect in distant tissues and in F1 progeny, indicating that cells may have an RNA-transport mechanism³. Similarly, two groups of researchers have demonstrated the systemic spread of co-suppression in plants^{12,13}. An RNA molecule, spreading throughout the plant via phloem, has been proposed as the mobile agent responsible for transmitting the co-suppression state¹².

Possible mechanisms for RNA-mediated interference

The sub-stoichiometric activity of the interfering RNA in *C. elegans* led to various models: that interference involves a catalytic mechanism dependent on the injected RNA; that the input material is amplified; or that interference occurs at the level of the gene. Several lines of evidence argue against DNA in the genome as a target for RNAi. Effects of dsRNA are generally not heritable beyond the first generation; injected animals and progeny exhibit the effects of RNAi, whereas animals of the F2 generation generally revert to a wildtype phenotype³. Additional evidence comes from direct sequencing of genomic DNA following RNA-mediated interference with *unc-22*; these experiments yielded no indication of mutations in the target gene (S. Xu and A. Fire, unpublished). Consistent with an RNA target, interference was effective using a variety of regions present in mature RNA, but was not effective using intronic or promoter sequences³. At this point, one attractive hypothesis is that dsRNA might result in early degradation of the endogenous mRNA. We know from *in situ* hybridization studies that RNA transcripts of a target gene fail to accumulate after RNAi (Ref. 3). Conceivably, the lack of mRNA products could be an indirect consequence of blocked processing or transport. Alternatively, endogenous transcripts could be degraded by a sequence-specific mechanism directed by dsRNA.

The mechanisms mediating certain co-suppression phenomena in plants have been shown to act both on the DNA template and on RNA products. Wassenegger *et al.*⁶ showed that viral or transgene-generated RNA could direct *de novo* modification (presumably methylation) of a homologous sequence in the plant genome. Other studies provide cases in which RNA-mediated co-suppression acts

post-transcriptionally, potentially by the rapid degradation of the target transcript¹⁴. In one example, transgene-mediated silencing of the endogenous gene encoding β -1,3-glucanase in tobacco, de Carvalho Niebel and colleagues¹⁵ demonstrated that the suppressed genes are actively transcribed. Subsequently, Jacobs *et al.*¹⁶ showed that gene silencing in this line correlates with an increased turnover of both the transgenic and the endogenous transcripts of β -1,3-glucanase. In the case of virally provided RNA sequences, it appears that viral RNA molecules can serve as targets as well as 'triggers' for co-suppression^{8,13,17}. Could there be a mechanistic link between (a) RNA-mediated degradation of RNA and (b) RNA-mediated methylation of DNA? One possibility is that these are two separate processes mediated by similar RNA molecules; alternatively, there could be a causal relationship, perhaps from an ability of RNA decay products to trigger methylation of homologous sequences in replicating DNA.

Does RNA-mediated interference do a job for the cell?

In addition to the mechanistic questions, attention is also merited to the physiological role for the RNA-associated silencing phenomenon. A role for co-suppression mechanisms in systemic defense against viruses has been suggested for plants^{17,18} and could apply to other organisms as well. Such a response represents an effective means by which to prevent viral replication and induce resistance in surrounding tissues prior to viral invasion.

Alternatively, co-suppression/RNAi might modulate normal gene expression. One can easily imagine double-stranded RNAs being used by the cell as a potent means to turn off specific genes in response to physiological or developmental cues. Perhaps the best way to identify these processes will be to find mutants that are defective in carrying out RNAi.

Do RNA-interference mechanisms have counterparts outside of plants and nematodes?

Mammalian cells exhibit a global antiviral response to double-stranded RNA. In this response, the PKR protein kinase recognizes dsRNA and

unleashes a vehement but somewhat non-specific response leading to general translational arrest¹⁹. Intriguingly, this type of systemic response can occur if the dsRNA is provided extracellularly²⁰ (consistent with the possibility of dsRNA uptake by mammalian cells). Viruses have evolved a number of strategies for evading or inhibiting the PKR response²¹. Certain tissue-culture cell lines lack PKR and are susceptible to mutant viruses that would otherwise be non-virulent. Any gene-specific interference by dsRNA in PKR-proficient mammalian cells would be dependent on a transient lapse in the PKR response, or on a controlled level of dsRNA that was incapable of activating PKR.

A wealth of information indicates that specific RNA-mediated interference mechanisms contribute to the control of gene expression in vertebrate and other systems. For many of these contributions, the precise nature of the interfering RNA (single-stranded versus double-stranded material) has yet to be characterized. Antisense transcripts have been reported for large numbers of vertebrate genes²². In some cases, roles for these transcripts in regulating the sense transcripts from the opposite strand have been demonstrated. From an informatics perspective, a surprisingly large fraction of vertebrate mRNAs contain long-conserved sequences within the 3' untranslated region as well as long blocks without silent changes in their protein-coding regions²². Lipman²² has proposed that these conserved sites are regulatory targets of endogenous antisense transcripts encoded by the complementary strand of the gene. Such a mechanism would, thus, be common and relatively conserved. Endogenous genes regulated by antisense transcripts have also been described for the primitive eukaryote *Dictyostelium*, and such mechanisms have been studied in detail in Eubacteria and Archaeobacteria (reviewed in Ref. 23). Co-suppression phenomena, similar to that described for plants, have also been observed in *Dictyostelium*²⁴. It will be interesting in the next few years to learn whether any or all of these effects share underlying mechanistic features and we suggest, moreover, that by studying the mechanisms underlying these phenomena, we will be better able to interpret the native language of the cell.

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Appearance of Interferon Inducibility and Sensitivity during Differentiation of Murine Teratocarcinoma Cells in Vitro

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Summary

Pluripotent embryonal carcinoma (EC) cells do not produce interferon after treatment with a wide variety of inducers, nor are they sensitive to its action. Several differentiated lines derived from the EC cells, however, both produce and are sensitive to mouse interferon. Differentiation of EC cells in vitro is accompanied by development of interferon inducibility and sensitivity.

Introduction

The stem cells of mouse teratocarcinomas are embryonal carcinoma (EC) cells (Kleinsmith and Pierce, 1964). Cloned EC cells will differentiate when injected into an adult host (Pierce, Dixon and Verney, 1960) when injected into a blastocyst (Papaioannou et al., 1975; Illmensee and Mintz, 1976) and when cell culture conditions are changed (reviewed by Martin, 1975). The differentiation of EC cells in culture provides an opportunity for studying the changing susceptibility of cells to virus infection as their phenotype changes (Swartzendruber and Lehman, 1975; Kelly and Boccara, 1976). The EC cells are resistant to infection by polyoma and SV40, and susceptible to infection by adenovirus 2, while the differentiated cells produced by EC cells can be infected by all three viruses. We have investigated whether or not the EC cells and their differentiated products can produce interferon.

Results

Induction of Interferon

A number of different murine teratocarcinoma cells were treated with the interferon inducer, Newcastle Disease virus (NDV), and the interferon yield was

titrated by a standard interferon assay. We have used three clonal EC cell lines [247-DESCI₂, 299-3 (see Experimental Procedures) and OC15S1 (McBurney, 1976)] and have also followed the change in phenotype as OC15S1 cells differentiated in culture. In addition, EC cells were isolated directly from embryoid bodies grown in animals (T76-12A and B); these were also allowed to differentiate in culture and tested for their capacity to produce interferon. We also studied several cloned differentiated cell lines derived from teratocarcinomas; these were parietal yolk sac cells (PYS-2), fibroblasts (LTP) and a nontumorigenic line SV₄₀-Cl₂ (Lehman et al., 1974; Swartzendruber and Lehman, 1975).

Table 1 shows that EC cells cannot be induced to produce interferon, while cultures containing differentiated derivatives of EC cells can be induced to do so. The failure of the EC cells to produce interferon was not due to the failure of the virus to adsorb or replicate sufficiently to induce interferon (Sheaff, Meager and Burke, 1972), since an extensive cytopathic effect was seen in the infected cells which was a result of virus infection. It was possible, however, that the virus destroyed the cells too rapidly for interferon to be formed. We therefore measured the amount of interferon formed by treatment of the EC cells with a wide variety of inducers (Table 2). These were poly(rI.rC) with and without DEAE-dextran (DEAE-DX), two types of superinduction conditions (Vilcek and Ng, 1971; Mozes and Vilcek, 1975), two replicating and one nonreplicating virus, and both virus and poly(rI.rC) in cells primed with interferon (Stewart, Gosser and Lockart, 1971). In no case was any interferon formed. Since this range of inducers represents examples of all the different agents known to be active in tissue culture, we concluded that EC cells could not be induced to form interferon; nor was there any constitutive production of interferon by any of the cell lines—that is, in the absence of any inducer.

To establish that the appearance of inducibility was not due to the selection of a minor subpopulation of cells in the EC cell cultures, we studied inducibility under conditions in which the EC cells differentiate with little cell division (Experimental Procedures). It can be seen that under these conditions, interferon inducibility develops from a low initial level to one typical of the differentiated cells (Table 3). It is not possible to state with confidence whether the yield of 4 interferon units per 10⁶ cells on day 1 is due to interferon or to a small nonspecific depression of virus RNA synthesis.

The resistance of EC cells to polyoma and SV40 virus infection (Swartzendruber and Lehman, 1975) could have been due to interferon production. The

EC cells, however, neither produce interferon constitutively nor can they be readily induced to do so (Tables 1 and 2). It remained possible that the EC cells were, for unknown reasons, already in a hyporesponsive state for interferon production. In this state (usually produced by treatment of cells with an interferon inducer), cells initially produce interferon and are protected from virus infection by its action. Such cells are subsequently unable to produce more interferon when exposed to a second inducer (Breinig, Armstrong and Ho, 1975).

The following experiment shows that the EC cells are not in the hyporesponsive state and that they remain fully susceptible to infection by viruses which are known to be interferon-sensitive. Semliki Forest virus (SFV), rather than NDV, was used to measure the susceptibility of the cells to virus infection because of its greater sensitivity to interferon action. Cells were infected at a multiplicity of 10 pfu per cell, and virus growth was followed by plaque titration of released virus and also by

measuring the amount of viral RNA synthesis by pulsing with ^3H -uridine after treatment with actinomycin to suppress host cell RNA synthesis. The results (Figure 1) show that virus grew well in all four cell lines—in fact, rather better in the lines that could not produce interferon (247-DESCI₂ and 299-3) than those that could (SV40-Cl₂ and PYS-2). The virus also grew well and induced the production of interferon in another differentiated cell line, LTP (data not shown). We concluded that there was no evidence that the EC cells were in the hyporesponsive state. The production of virus RNA in infected cells meant that the virus RNA reduction assay, which we have used routinely for interferon titrations (Atkins et al., 1974), could be used to

Table 1. Interferon Induction in Mouse Teratocarcinoma Cells by NDV (Strain B1)

Cell Line ^a (and Number per Culture)	Interferon Titer (log ₁₀ Units)	Interferon Titer/10 ⁴ Cells
Stem cells (embryonal carcinoma)		
247-DESCI ₂ (4 × 10 ⁵)	<0.5	<8
Unipotential stem cell line		
299-3 (2.5 × 10 ⁵)	<0.5	<1
Partially differentiated cell cultures		
T-76-12 A (2.0 × 10 ⁵)	3.20	793
T-76-12 B (3.9 × 10 ⁵)	2.55	910
Differentiated cell lines		
PYS-2 (9.2 × 10 ⁴)	3.75	6110
SV40-Cl ₂ (8 × 10 ⁵)	3.40	3140

^a (247-DESCI₂) 95–99% pure EC cells; (299-3) 95–99% pure EC cells; (T-76-12 A) 60% differentiated cells; (T-76-12 B) 60% differentiated cells.

Table 2. Attempted Interferon Induction in EC Cells (DESCI₂)^a

Inducer	Interferon Titer (log ₁₀ Units)
Poly(rI.rC) (50 μg/ml)	<0.5
Poly(rI.rC) (50 μg/ml) + DEAE-DX (100 μg/ml)	<0.5
Poly(rI.rC) (50 μg/ml) followed by cycloheximide and actinomycin ^b	~0.25
Ultraviolet irradiation followed by poly(rI.rC) (50 μg/ml) ^c	~0.25
NDV (2.6 × 10 ⁶ pfu) ^d	<0.5
Ultraviolet-NDV (ultraviolet-irradiated to 8 × 10 ⁴ pfu)	<0.5
Semliki Forest virus (1.9 × 10 ⁶ pfu)	<0.5
NDV (2.6 × 10 ⁶ pfu) in cells primed with 10, 50 or 100 units of interferon	All <0.5
Poly(rI.rC) (50 μg/ml) in cells primed with 10, 50 or 100 units of interferon	All <0.5

^a 4.0 × 10⁵ cells per culture.

^b Using the conditions of Havell and Vilcek (1972).

^c Using conditions determined to be optimal for human foreskin fibroblasts as described by Mozes and Vilcek (1975).

^d Strain B1.

Table 3. Interferon Inducibility of Differentiating EC Cells

Cell Treatment	Cell Number	Interferon Titer (log ₁₀ Units)	Interferon Titer/10 ⁴ Cells
Maintained at high density	1.83 × 10 ⁶	0.90	4
Induced first day at low density	4.15 × 10 ⁵	<0.5	<7
Induced second day at low density	5.14 × 10 ⁵	1.0	20
Induced third day at low density	2.69 × 10 ⁵	1.9	300
Induced fourth day at low density	3.0 × 10 ⁵	2.20	550
Induced fifth day at low density	3.95 × 10 ⁵	2.95	2270
Induced sixth day at low density	3.0 × 10 ⁵	3.10	4230

EC cells (OC15S1) were allowed to differentiate in culture and were interferon-induced each day with NDV as inducer, as described in Experimental Procedures. The interferon titer is the average of two determinations on each day; similar results were obtained in a repeat of this experiment.

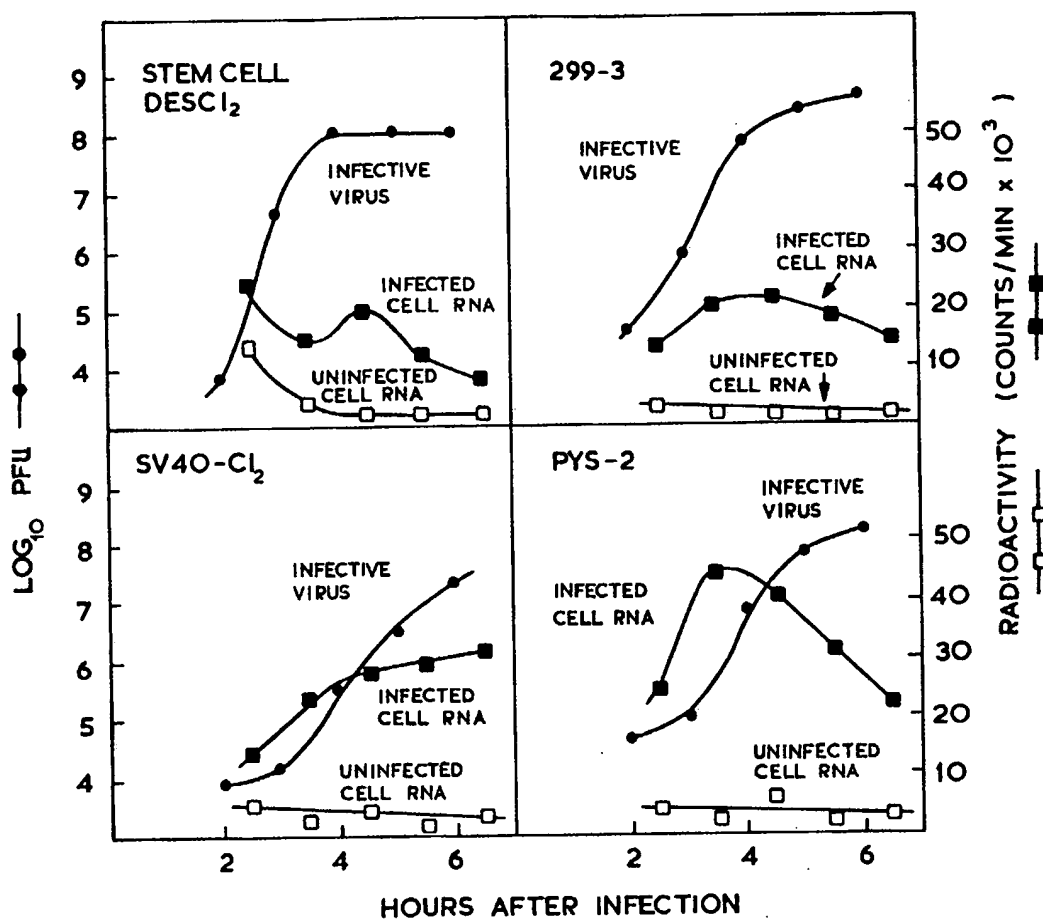


Figure 1. Multiplication of Semliki Forest Virus in Teratocarcinoma Cells
Cells were infected at a multiplicity of ~ 10 , and virus RNA synthesis and virus infectivity were determined as described in Experimental Procedures. The cultures of 299-3, PYS-2 and SV40-Cl₂ contained about 2×10^6 cells in a 3.5 cm petri dish; those of DESC₁₂ contained about 2×10^5 cells in the wells of a Linbro disposable tray. In the latter case, the radioactive counts have been multiplied by 10 to correct for this difference.

determine how sensitive the various cell lines were to the antiviral effect of Interferon. In this experiment, we used L cells and various teratocarcinoma cell lines (Table 4). These cells were exposed to a high titer preparation of interferon to determine whether this would protect the cells from virus infection and replication. The action of interferon was measured by noting its effect on virus RNA synthesis after infection by SFV, an interferon-sensitive virus. If the cells were insensitive to the action of interferon, then they would not be protected against the virus and the added interferon would appear to have no effect; this would be described as a low interferon titer. Conversely, if the cells were protected by interferon, then the interferon would appear active and it would be

described as possessing high titer. Both EC cells (247-DESC₁₂) and the 299-3 cell line were not protected from virus infection and replication by interferon. These were described as insensitive to the action of interferon despite the fact that there is a slight effect of the added interferon on virus replication, as shown by the low rather than zero titers. These low titers are probably due to nonspecific effects of the unpurified mouse interferon which cause a small reduction in viral RNA synthesis. Note that the differentiated cell lines and L cells are protected by interferon against virus infection and replication by almost the same extent. This is shown by the high interferon titers in Table 4. The insensitivity of the 299-3 cell line to interferon was confirmed by measuring the plaque size of Semliki

Table 4. Interferon Sensitivity of Various Mouse Teratocarcinoma Cells and L Cells

Cell Line	Titer of a Single Preparation of Mouse Interferon When Titrated in the Different Cell Lines (log ₁₀ Units)
L	4.80
Embryonal Carcinoma DESCI ₂	0.30
299-3	0.90
Differentiated Cell Lines PYS-2	3.45
SV40-Cl ₂	4.30
LTP	4.20

The interferon titer is a measure of the ability of added interferon to inhibit the replication of Semliki Forest virus. Since the titer is the reciprocal of the dilution reducing viral yield to 50% of controls, low figures demonstrate that added interferon is poor at reducing viral replication, and that these cells have a reduced sensitivity to the protective effect of interferon.

Forest virus, since interferon production is known to be one of the factors governing plaque size (Fauconnier, 1970). The experiment could not be performed with the EC cell line DESCI₂ because the cells could not be grown out to a confluent sheet before differentiation took place. The results (Table 5) showed that the virus caused much larger plaques in 299-3 cells than in the cell lines that produced interferon, and therefore further suggest that interferon is not produced by 299-3 cells.

To establish that interferon sensitivity was not due to selection of a minor population of cells in the EC culture, a single sample of interferon was titrated in a series of cultures of the OC15S1 line of EC cells, in which differentiation takes place accompanied by little cell division. The results (Table 6) showed that the EC cells were almost completely insensitive to mouse interferon, and that a culture which had been allowed to differentiate for 2 days had a sensitivity intermediate between that of the EC cells and that of the final culture of epithelial cells.

Discussion

Only a few cells of mammalian or avian origin have previously been shown to be unable to produce interferon, and this is the first report of a cell that is insensitive to interferon. Several cell lines, including Vero (African green monkey), BHK/21 and CHO-K1 cell lines have been shown to be incapable of producing interferon although they are all sensitive to its action (Desmyter, Melnick and Rawls, 1968; Taylor-Papadimitriou and Stoker, 1971; Morgan, 1976).

We have shown that embryonal carcinoma cells must contain the genes responsible for both the

Table 5. Plaque Size of Semliki Forest Virus in Mouse Teratocarcinoma Cells

Cell Line	Virus Titer	Plaque Size
299-3	2.9×10^7	4.0 mm
PYS-2	1.07×10^7	1.5 mm
SV40-Cl ₂	6.2×10^6	1.0 mm
LTP	1.49×10^7	1.5 mm

A single stock of virus was used.

Table 6. Interferon Sensitivity of Differentiating EC Cells

Cell Treatment	Titer of a Single Preparation of Mouse Interferon (log ₁₀ Units)
Cells maintained at high density	0.60
Interferon added second day at low density	2.7
Interferon added fourth day at low density	3.7
Interferon added sixth day at low density	3.65

EC cells (OC15S1) were allowed to differentiate in culture, and a single preparation of interferon was titrated after incubation on the days shown. This preparation of mouse interferon had a titer of 4.5 log₁₀ units in L cells.

induction and action of interferon, but in a repressed state, and that the genes in question are inducible in the differentiated cells, whether in pure culture or in a mixed culture along with EC cells. Thus the process of differentiation into cells which resemble embryonic endoderm is accompanied by the activation of the interferon system, and the production of both sensitivity to interferon can be used as a marker in studies on differentiating teratocarcinoma cells (compare Adamson, 1976). The EC cells supported the growth of Semliki Forest well, the virus growing to slightly higher titers than in the differentiated lines, possibly because of the lack of an active interferon system. The reported failure of SV40 and polyoma virus to infect these cells is not due to the endogenous production of interferon, and the block must lie elsewhere.

Two groups have recently shown that EC cells were resistant to ecotropic C-type viruses, but that the viruses will multiply in the differentiated cell lines. Peries et al. (1977) found that neither murine sarcoma virus nor murine leukemia virus would replicate in cells, but that both would replicate in the derived differentiated cells, while Teich et al. (1977) also found that EC cells were resistant to infection by murine leukemia virus, although they were sensitive to infection with viruses whose replication does not involve integration, such as

EMC, Sindbis, VSV and vaccinia viruses. In contrast, the differentiated cells (excluding endoderm) were susceptible to murine leukemia virus, although the number of cells infected and the yields of virus were extremely low.

The failure to activate genes responsible for the interferon system and the failure of tumor viruses to integrate suggest that in both cases, the genome is masked in some way.

Experimental Procedures

Cells

The cell lines were 247-DESCI₂ and OC15S1, multipotential carcinoma cell clones; 299-3, an embryonal cell clone; T76-12A and B, two embryonal carcinoma cell lines; PYS-2, a parietal yolk sac cell clone; LTP, a differentiated line of fibroblasts; and SV40-Cl₂, a cell clone derived from SV40-infected, mixed teratocarcinoma (embryonal carcinoma and differentiated cells) (Lehman et al., 1974; Swartzendruber and Lehman, 1975).

The 247-DESCI₂ was maintained in culture as nearly (95-99%) pure embryonal carcinoma; if allowed to differentiate, however, these cells are capable of producing a multiplicity of differentiated cells and tissues. The 247-DESCI₂ cells used in these experiments were pure embryonal carcinoma, since the cells were near diploid (Swartzendruber, Cram and Lehman, 1976), produced teratocarcinomas when injected into adult 129/J strain mice and were not infected (measuring T antigen, V antigen and infectious virus) with either SV40 or polyoma viruses. The 299-3 line produced teratocarcinomas upon injection into 129/J strain mice, but the differentiated tissues were restricted to neuroepithelium. A majority of the cells of this line were resistant to infection with SV40 and polyoma viruses. This line retains numerous characteristics of the embryonal carcinoma cells, but is restricted in its potential to differentiate. The two differentiated lines, LTP and PYS-2, did not produce teratocarcinomas when inoculated into animals. If the animals were irradiated (500 r), however, a few tumors were produced, a fibrosarcoma (LTP) and parietal yolk sac tumor (PYS-2). The PYS-2 line produced basement membrane (Lehman et al., 1974). Both lines are tetraploid, and susceptible to SV40 and polyoma virus infection. In the line SV40-Cl₂, approximately 100% of the cells were positive for SV40 T antigen; the cells are tetraploid and not capable of producing teratocarcinomas when injected into adult animals. Since SV40 is capable of infecting only the differentiated cells (Swartzendruber and Lehman, 1975), this indicates that these cells were derived from a differentiated cell population. Two lines (T76-12 A and B) were developed from embryoid bodies obtained from the ascites of an intraperitoneal solid teratocarcinoma. These cells were allowed to differentiate and contained >60% differentiated cells in the population as determined by the above criteria.

OC15S1 was maintained by subculturing daily by plating 2×10^6 cells per 50 mm dish. The cells of OC15S1 with EC cell morphology form teratocarcinomas with ciliated and glandular epithelia and with cartilage (McBurney, 1976). In culture, they form striated beating muscle (C. F. Graham, unpublished data) and nerve (Adamson, 1976). They are therefore pluripotent, tumorigenic, embryonic stem cells which resemble other EC cell lines. For differentiation, these cells were incubated in PBS (solution A of Dulbecco and Vogt, 1954) containing 0.5 mM EGTA at 37°C for 30 min. After vigorous pipetting, the majority of cells are single (at least 95%) and are plated at low density (2×10^5 cells per 50 mm dish) on a gelatin-coated dish (Yaffe, 1973) with 2 ml of α -modified medium (Stanners, Eliceiri and Green, 1971) lacking nucleosides and deoxynucleosides and containing 5% (v/v) heat-inactivated calf serum and 0.05 mM dibutyl cyclic 3'-5'-adenosine monophosphate (Sigma Chemical Company, Pound Lane, London). The medium was preequilibrated with 5%

CO₂ in air and changed every 2 days. Under these conditions, there is some cell death during the first 2 days of culture (up to 50% death), but subsequently there is little or no cell death, and the cell number increase is very slow, approximately doubling during the following week. At the end of this period, about 90% of the cells look like epithelial cells. L cells were maintained in Eagle's minimum essential medium (EMEM) plus 10% fetal calf serum (FCS). Chick embryo cells were prepared as described by Morser, Kennedy and Burke (1973).

Viruses

NDV (strains B1 or F) was grown in fertile 10 day old hen eggs, and infectivity was titrated in primary chick embryo cells. UV-NDV was prepared by irradiating 1 ml aliquots of virus with 720 erg/mm² from a General Electric germicidal lamp (G30TS) after overnight dialysis against phosphate-buffered saline. SFV was grown in chick embryo cells in suspension (Kennedy and Burke, 1972) and titrated in primary chick embryo cells.

Production and Assay of Interferon

Interferon was induced by treatment with virus or poly(rI.rC) for 1 hr at 37°C then followed by washing with EMEM plus 2% FCS and incubation in the same medium at 37°C for 24 hr before harvesting the fluids. Samples for interferon assay induced by viruses were dialyzed for 5 days (NDV and UV-NDV) or 1 day (SFV) against a pH 2 buffer to destroy virus, before dialysis against Earle's buffered salt solution overnight. Interferon was assayed by a micro-modification of the viral RNA reduction assay (Atkins et al., 1974) in which five serial 0.5 log₁₀ dilutions of interferon were incubated overnight with 10⁶ L cells in small glass vials, before challenge with 250 μ l of SFV (1×10^7 pfu) in the presence of actinomycin (1 μ g/ml) and the addition of 250 μ l of ³H-uridine (final concentration 1 μ Ci/ml) 2 hr later. This concentration of actinomycin was sufficient to depress cellular RNA synthesis to 22% of control and thus make it possible to measure viral RNA synthesis. The assay was terminated 3 hr after the addition of the isotope by withdrawal of the fluids and washing with ice-cold 0.9% NaCl, ice-cold 5% TCA (twice) and 95% EtOH. The dried cell sheets were dissolved in 100 μ l Soluene (diluted 1:3 with toluene) before the addition of 2 ml of scintillation fluid and counting. The interferon titer was defined as the reciprocal of the dilution causing a 50% depression in virus RNA synthesis. The results are expressed in International reference research units and all assays contained an internal standard. Interferon assays in mouse teratocarcinoma cells used Linbro trays since the cells, particularly the EC cells, adhered better to the plastic. The dried cell sheets were dissolved in 500 μ l of 0.25 M NaOH, and 250 μ l were added to a suitable scintillator before counting.

Virus Growth in Teratocarcinoma Cells

Cells (2×10^6 in a 3.5 cm petri dish, or 2×10^5 in a Linbro disposable tray) were infected with SFV (500 μ l per petri dish at a multiplicity of 10; 250 μ l per Linbro tray at a multiplicity of 50) for 1 hr at 37°C in EMEM plus 2% FCS and 1 μ g/ml actinomycin. The cells were then washed twice with EMEM plus 2% FCS and 1 μ g/ml actinomycin before incubation with 2 ml per petri dish or 500 μ l per Linbro tray of the same medium. At intervals, the fluids were withdrawn and stored at -70°C for subsequent plaque assay, and ³H-uridine (2 μ Ci/ml) in EMEM + 2% FCS and 1 μ g/ml actinomycin were added (500 μ l per petri dish or 250 μ l per Linbro tray). After 1 hr, the cell sheets were washed, dried, dissolved in 0.25 M NaOH and counted as described for the interferon assay.

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